Université de Montréal

Études des mécanismes d'induction de l'immunosuppression par le virus Herpès Humain 6

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Résumé

HHV-6 est un virus herpès humain ubiquitaire. La plupart des individus deviennent séropositifs à l'âge de 2 ans. L'infection primaire par HHV-6 donne lieu à une maladie fébrile chez les enfants, appelée exanthème subitum ou la roséole. Par contre, chez l'adulte, cette infection cause des maladies de type mononucléose. L'infection reste généralement latente chez les individus sains, mais elle se réactive souvent chez les personnes immunodéprimées, par exemple, chez les personnes greffées et les patients atteints du sida. HHV-6 a été associé à plusieurs types de cancers et de désordres lymphoprolifératifs. Ce virus induit l'immunosuppression et inhibe la prolifération des lymphocytes par les mitogènes. C'est pour toutes ces raisons que nous voulions savoir si ce virus dérègle le cycle cellulaire des cellules qu'il infecte. Les travaux réalisés durant cette thèse ont porté sur les changements induits dans les cellules humaines par ce virus au cours de la progression du cycle cellulaire.

Nous avons montré que l'infection par HHV-6 retarde la progression du cycle cellulaire dans la lignée cellulaire T humaine HSB-2, ainsi que dans les lymphocytes T primaires humains pour les accumuler dans les phases S et G2/M. Cependant, après avoir traité les cellules avec la nucléase du Micrococcus, nous avons constaté que le cycle cellulaire des cellules infectées s'accumulait plutôt dans la phase G2/M. La nucléase dégrade préférentiellement l'ADN virale. Nous avons observé une augmentation de l'activité kinase de cdc2 dans les cellules infectées malgré une baisse des niveaux de ses partenaires catalytiques, la cycline A et la cycline B. Nos études ont montré qu'il y a une diminution drastique de la protéine p21 dans les cellules infectées, en dépit de la stabilisation et de l'activation de p53 induite dans ces cellules. Ce qui laisse penser que la protéine p53 pourrait être inactive sur le plan transcriptionnel dans les cellules infectées. Cette diminution de p21 dans les cellules infectées est partiellement restaurée après incubation des cellules dans un milieu de culture contenant des inhibiteurs du protéasome. En plus, nous démontrons ici qu'une protéine virale précoce, p41, s'associe et se fixe avec cdc2 et augmente son activité kinase. Tous ces résultats suggèrent que HHV-6 provoque des perturbations énormes dans la progression normale du cycle cellulaire dans les cellules T humaines. Dans ces études, nous avons démontré aussi que l'infection par HHV-6 induit l'autophagie dans les cellules HSB-2, comme il a été démontré par l'induction de LC-3 II et par la formation de vacuoles autophagiques dans les cellules qui sont infectées. Nos résultats indiquent que HHV-6 inhibe la fusion entre les vacuoles autophagiques formées et les lysosomes dans les cellules infectées modulant ainsi la réponse autophagique des cellules hôtes infectées. Nous avons trouvé aussi que

l'inhibition de ce processus par un inhibiteur pharmacologique diminue la réplication virale. L'autophagie est un processus physiologique cellulaire pendant lequel les vieux constituants cellulaires (mitochondries, protéines cellulaires, etc) se dégradent. Le fait que ce processus soit modulé dans les cellules dépendantes des différentes phases du cycle cellulaire, nous a poussé à l'étudier. Enfin, nous essayons d'investiguer la réplication virale dans les cellules dépourvues de *p53*, le gène suppresseur de tumeur, qui contrôle la progression de cycle cellulaire. Nous avons émis l'hypothèse suivante, que ces virus peuvent mieux se répliquer dans les cellules n'exprimant pas le gène *p53*. En vérifiant cette hypothèse, nous avons trouvé que l'inhibition de l'expression de p53 provoquée par siRNA ou par un agent pharmacologique conduit à une mort cellulaire massive dans une lignée de cellules T humaines ayant un gène *p53* de type sauvage. Nous démontrons que cette mort se produit aussi dans une autre lignée cellulaire dont le *p53* est muté et qu'elle pourrait être évitée par des inhibiteurs d'autophagie ou de nécroptose. Nos observations mettent en évidence qu'un niveau d'expression basale de *p53* est nécessaire à la survie cellulaire.

Abstract

HHV-6 is a ubiquitous human herpesvirus. Most individuals become infected at the age of 2 years. Primary infection by the virus causes a self-limiting febrile illness called exanthem subitum or roseola. In adults, primary infection may cause mononucleosis-like illnesses. The infection usually remains latent in healthy individuals, but often reactivates in immunocompromised individuals, for example, transplant patients and AIDS patients. The virus has also been associated with cancers and lymphoproliferative disorders. The virus encodes two proteins that interact with p53. However, little is known concerning the impact of the virus on cell cycle progression in human cells. The investigations reported in the thesis were focused on this issue.

We show here that that HHV-6 infection delays the cell cycle progression in human T cell line HSB-2, as well as in primary human T cells and causes their accumulation in S and G2/M phase. By degrading the viral DNA in the virus-infected cells, we show that the infected cells accumulate in the G2/M and not in the S phase. We observed an increase in the kinase activity of cdc2 in virus-infected cells despite lower levels of its catalytic partners, cyclin A and cyclin B. We show here that the viral early antigen p41 associates with, and increases the kinase activity of, CDK1.

Our studies have shown that there is a drastic reduction of p21 protein, despite the virus-induced stabilization and activation of p53 suggesting that p53 may be transcriptionally inactivated in the virus-infected cells. This decrease of p21 in infected cells was partially restored by proteasome inhibitors. These results suggest that HHV-6 causes perturbations in the normal progression of cell cycle in human T cells.

Autophagy is a physiological cell process during which old cellular constituents and long-lived proteins in cells are degraded. This process is regulated in a cell cycle-dependent manner. We show here that infection with HHV-6 induces autophagy in HSB-2 cells. This was shown by the induction of LC-3 II as well as by the appearance of autophagic vacuoles in the virus-infected cells. However, we found that the virus inhibits fusion between autophagic vacuoles and lysosomes formed in infected cells, thus evading the autophagic response of infected host cells.

Finally we tried to investigate replication of the virus in human cells in the absence of P53; a tumor suppressor gene which is also known as "the guardian of the genome ".

During these investigations, we found that that inhibition of p53 gene expression mediated by siRNA as well as its inhibition by pharmacological inhibitors leads to massive cell death in human T cell line HSB-2 that carries a wild-type

p53. We show that this death also occurs in another cell line CEM, which carries a transcriptionally mutated p53.
Interestingly, the cell death could be prevented by pharmacological inhibitors of autophagy and necroptosis.
Taken together, our results provide important novel insights concerning the impact of HHV-6 on cell cycle regulation and autophagy as well as of basal level p53 in cell survival.

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Seul le silence est grand, tout le reste est faiblesse.

-Les Destinées-Vigny Alfred

Les gens qui ont peu d'affaires sont de très grands parleurs: moins on pense, plus on parle.

-Montesquieu

Savoir où l'on veut aller, c'est très bien; mais il faut encore montrer qu'on y va

-Zola Émile

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ADN: Acide Désoxyribonucléique

AIP1: Apoptosis Inducing Protein-1

APC: Anaphase Promoting Complex

APC/C: Anaphase Promoting Complex/Cyclosome

ATRIP: ATR (Ataxia Telangiectasia-mutated Related)-Interacting Protein

BAD: Bcl-2-associated death promoter

BAK: Bcl-2-homologous antagonist/killer

Bcl-2: B cell lymphoma protein-2

Bid: BH-3-interacting domain death agonist

BL: Lymphome de Burkitt

BARTs: Bam H1 A-fragment Rightward Transcripts

Bax: Bcl-2-associted X protein

CAK: CDK-Activating Kinase

CBP: CREB Binding Protein

CCC: Checkpoint Clamp Complex

Cdc: Cycle de division cellulaire

CDK: Kinases Cycline-Dépendantes

CDKI: CDK Inhibiteurs

Cdt1: CDC10 dependent transcript 1

CEN: Centromeric Protein

CHK: Checkpoint Kinase

CHMP-1B: Chromatin-Modifying Protein 1B

CHMP-4B: Chromatin-Modifying Protein 4B

Cip-1: CDK-interacting protein (p21)

CITK: Citron Kinase

CK-2: Casein kinase

Cks: Cyclin kinase subunit

CPC: Chromosome Passenger Complex

CRL: Cullin-RING Ubiquitine E3 Ligases

CTL: Lymphocytes T Cytotoxiques

CS: Centralspindlin

DHFR: Dihydrofolate Réductase

DNA-PK: Protéine Kinase Dépendante de l'ADN

DP-1/2: Differentiation-regulating transcription factor-1/2

DPPF: DNA Polymerase Processivity Factor

DRAM: Damage Response Autophagy Modulator

DUB: Déubiquitinase

E: Early (proteins précoces)

E6AP: E6-associated protein

EBER: EBV (Epstein-Barr virus)-Expressed RNA

EBNA: Antigène Nucléaire du virus d'Epstein-Barr

EBV: Virus d'Epstein-Barr

ECT-2: Epithelial Cell Transforming gene 2

Emi-1: Early mitosis inhibitor-1

ESCRT: Endosomal Sorting Complex Required for Transport

FBW: F box and WD repeat domain containing protein

FYVE-CENT: Fab-1, YOTB, Vac-1 and EE-1-domaine

GAK: G-Associated kinase

GAP: GTPase-Activating Protein

GEF : Facteur d'Echange du nucléotide Guanine

GSK -3β: Glycogène synthase kinase 3beta

HCMV: Human Cytomegalovirus

HDAC: Histone-Désacétylases

HECT: Homologous to E6-associated protein C Terminus

HHV-6: Herpèsvirus humain de type 6

HHV-7: Herpèsvirus humain de type 7

HMG-1: High Mobility Group-1

HPV: Human Papillomavirus

HPVE7: Human Papillomavirus E7

HSV-1: Herpès simplex virus-1

hSWI/SNF: SWItch/Sucrose Non-Fermenting

ICP: Infected Cell Protein

IE: Immediate early

INCENP: Inner Centromere Protein

Kbp: kilo paires de bases

KS: Kaposi's Sarcoma

KO: Knocked Out

LCL: Lignée Cellulaire Lymphoblastique

LMP: Latent Membrane Protein

3-MA: *3*-Méthyladénine

MAT-1: Ménage-à-trois

MAX: Myc-associated protein X

MCM: Mini-chromosome maintenance

MDM2: Mouse Double Minute2

MKLP: Mitotic Kinesin-Like Protein

MRLC: Myosine II Regulatory Light Chain

MT: Microtubules

mTOR: Mammalian Target Of Rapamycin

NK: Natural Killer

Nm: Nanomètre

NPC: Carcinome du Nasopharynx

ORC: Origin recognising complex

PBL: Leucocytes de sang périphériques (*Peripheral Blood Leukocytes)*

PCNA: Antigène Nucléaire de Prolifération Cellulaire

PCR: Réaction de polymérisation en chaîne

PERK: PKR (voir ci-dessous)-related Endoplasmic Reticulum Kinase b

PFT: Pifithrine

PH: Pleckstrin Homology

PI: Post Infection (dans le contexte d'une infection virale)

PI: Iodure de Propidium (dans le contexte de coloration des cellules)

PIG-3: P53-induced gene-3

PKR: Double stranded RNA-dependent Protein Kinase

PLK: Polo-Like Kinase

PML-NB: Promyelocytic Nuclear Bodies

PPA: Phosphonoacetic Acid

PP2A: Protein Phosphatase-2A

PUMA: P53 Upregulated Modulator of Apoptosis

PRb: Protéine du rétinoblastome

PRC1: Protein Regulator of Cytokinesis-1

P-TEFb: Positive-acting Transcription Elongation Factor

PyMT: Polyoma Virus Middle T

RIG-1: Gène-1 Induit par l'acide Rétinoïque

RING: Really Interesting New Gene

ROC: Regulator of Cullin

ROK: Rho kinase

RPA: Replication Protein *A*

USP: Ubiquitin-Specific Protease

SCC-1: Subunit-1 of cohesin complex

SCF: Skp-1/Cullin/F-box

Sdi-1: Senescent cell-derived inhibitor of DNA synthesis-1 (p21)

SIRT-1: Sirtuin-1

Smc: Structural Maintenance of Chromosome protein

SREBP: Sterol-Response Element Binding Protein

SIDA: Syndrome d'Immuno-Déficience Acquise

SV40LT: Simian Virus-40 Large-T

TAF-1: Template activating factor

TFIIH: Facteur de Transcription IIH

TFIIB: Facteur de Transcription IIB

β-TRCP: β-Transducin Repeat-Containing Protein

TSG-101: Tumor Susceptibility Gene 101

TTC-19: Tetratricopeptide repeat domain 19

VHC: Virus de l'Hépatite C

VIH: Virus de l'Immunodéficience Humaine

Vhs: Virion host shutoff

Waf-1: Wild type p53-activated factor-1 (p21)

Chapitre 1

1. Introduction

Avant-propos

Comme l'indique le titre de cette thèse, les études présentées dans ce manuscrit visent à comprendre le mécanisme d'immunosuppression induit par le virus herpès humain (HHV-6). L'un des principaux effets de l'infection virale est d'inhiber la prolifération des cellules humaines en réponse à divers mitogènes. Cette inhibition est probablement la principale cause d'immunosuppression. L'induction de l'inhibition de la prolifération cellulaire par ce virus suggère qu'il est capable d'affecter la progression du cycle cellulaire des cellules infectées. D'ailleurs, il est bien connu que d'autres virus herpès manipulent aussi la progression du cycle cellulaire dans le but de favoriser leur propre réplication. Au début de notre ouvrage, aucune information n'était pratiquement disponible dans la littérature scientifique concernant l'impact de HHV-6 sur la progression du cycle cellulaire dans les cellules humaines. Par conséquent, nous nous sommes intéressés à étudier la manière dont HHV-6 affecte la progression du cycle cellulaire dans les cellules humaines. Ainsi, nous présentons ci-dessous la littérature concernant HHV-6 et le cycle cellulaire.

1.1 Le Virus Herpes Humain 6 (HHV-6)

Le Virus Herpès Humain 6 (HHV-6) est le sixième membre de la famille des virus Herpès qui infectent les humains. Il a été découvert en 1986 dans des cultures de leucocytes de sang périphériques (PBL) provenant de patients présentant le syndrome d'immunodéficience acquise (SIDA) et des maladies lymphoprolifératives (1). HHV-6 est un virus ubiquitaire et des études sérologiques ont montré que plus de 90% des enfants sains en sont infectés dès l'enfance (2, 3). Ce virus est associé à plusieurs maladies aussi bien chez les personnes saines qu'immunodéprimées. Tout comme le HCMV (Cytomégalovirus humain), HHV-6 fait partie de la sous famille des Betaherpèsvirus et il est classé dans le genre des Roséolavirus avec HHV-7 (Virus Herpès Humain 7) (4). Il s'agit d'un virus enveloppé, à ADN double brin, faisant approximativement 167 kbp de longueur et un diamètre de 160-200 nm (5, 6). Les différents isolats et souches du HHV-6 se regroupent en deux variantes, HHV-6A et HHV-6B (7, 8). Les variantes A ont un tropisme plus neurotropique et infectent les cellules humaines T CD4+ et CD8+. Tandis que les variantes B qui se retrouvent fréquemment dans la salive et le sang infectent préférentiellement les glandes salivaires ainsi que les cellules T CD8+ (9, 10). Il existe seulement 4% de différence entre les séquences de HHV-6A et HHV-6B. La distinction entre les deux est possible par le polymorphisme de la longueur des fragments de restriction, les anticorps monoclonaux, par PCR ainsi que par les caractéristiques de

croissance in vitro (7, 8). Ces deux variantes différent aussi par leur sensibilité aux agents antiviraux, et par leurs tropismes pour les différentes cellules de l'hôte (11).

HHV-6 infecte préférentiellement les lymphocytes T CD4+ (12) mais il peut infecter également un grand nombre d'autres cellules avec moins d'efficacité, tel que les cellules B, les NK (*Natural Killer*), les monocytes, les cellules dendritiques et les CTL (lymphocytes T cytotoxiques), etc. Il a été démontré que la molécule CD46 agit comme étant un récepteur pour l'infection par HHV-6A et HHV-6B (13). HHV-6 cible principalement les lymphocytes et les monocytes, par conséquent, l'infection par ce virus induit des dérèglements importants au niveau du système immunitaire. Après l'infection, HHV-6 persiste dans le corps et établit la latence dans différentes cellules et organes comme les monocytes/macrophages, les glandes salivaires, le cerveau et les reins. Les infections latentes se réactivent chez les personnes immunodéprimées, transplantées et atteintes par le SIDA.

HHV-6 se transmet principalement par la salive et les habitudes de partage salivaire familiales influencent l'âge d'acquisition de l'infection (14). Malgré que la plupart des nouveau-nés soient protégés par les anticorps maternels, à l'âge de 6 mois, ils redeviennent séronégatifs et ils sont susceptibles à l'infection suite à la disparition progressive de ces anticorps (15). Les transmissions par voie nosocomiale pourraient avoir lieu dans les établissements hospitaliers lors des transfusions sanguines ou des transplantations d'organes et de moelles provenant de donneurs ayant le virus intégré dans leurs génomes (16). Des infections congénitales se produisent aussi dans 1-2% de cas chez les nouveau-nés engendré par l'intégration du génome viral dans le génome de l'un des deux parents, et sa transmission via les gamètes (17). Ce sont surtout les variantes du type A qui s'intègrent dans le génome (18). Toute fois, jusqu' à date, il n'existe aucune évidence qui suggère la transmission sexuelle de HHV-6.

L'infection primaire par HHV-6B cause une maladie infantile communément appelée la roséole ou encore la sixième maladie et qui affecte les enfants de moins de 3 ans (19). Cliniquement, il s'agit du syndrome d'exanthème subitum qui est l'une des principales causes des visites des salles d'urgences chez les enfants en bas âge. Cette maladie est caractérisée par une forte fièvre dont la durée est de 3-5 jours accompagnée d'otites et suivie d'une éruption cutanée (19-21). HHV-6 peut être aussi à l'origine du syndrome mononucléosique tout comme l'EBV (Virus d'Epstein-Barr) et le HCMV (22). Autres que la mononucléose, l'infection primaire par HHV-6 pourrait entrainer des lésions du type *papular-purpuric* qui caractérisent le syndrome de gants et chaussettes (23) ou encore celui de Gianotti-Crosti (24). HHV-6 est associé à plusieurs maladies graves dont la sclérose en plaques (25). Cependant, son implication dans des maladies malignes reste controversée et je site parmi lesquelles; la maladie de Hodgkin, le lymphome non hodgkinien, la leucémie lymphoblastique aigue, le carcinome oral ainsi le carcinome cervical (26-28).

De nos jours, il est bien connu que HHV-6 se réactive fréquemment chez les receveurs de transplantations, chez les patients atteints de cancer qui subissent la chimiothérapie ainsi que chez les patients infectés par le VIH (29). Ces réactivations se manifestent surtout par des fièvres très élevées (30), des éruptions cutanées, des pneumonies, des encéphalites et même le rejet des greffes (31). En effet, il a été montré que HHV-6 était à l'origine de la suppression des moelles osseuses et des cellules souches chez des patients greffés (32, 33). De plus, la réactivation de HHV-6 pourrait contribuer au développement du syndrome d'hypersensibilité aux médicaments (34, 35). C'est un syndrome qui a les mêmes symptômes systémiques que ceux de la mononucléose, et qui se caractérise par des éruptions cutanées, un dysfonctionnement hépatique et une lymphadénopathie.

Dans la majorité des cas, l'infection par HHV-6 chez les enfants se résorbe par elle-même et ne nécessite aucun traitement antiviral. Cependant, lors de la réactivation de ce virus chez les personnes immunodéprimées, plusieurs agents antiviraux ont été employés tel que le ganciclovir (36), le foscarnet (37) et le cidofovir (38).

De nombreuses études ont montré des associations entre HHV-6 et différents cancers et désordres lymphoprolifératifs (1, 26-28). Ce virus code pour au moins deux protéines qui s'associent à p53, une importante protéine suppresseur de tumeur, qui joue un rôle majeur dans, entre autres, la progression du cycle cellulaire (39). En outre, les virus en général et les virus à ADN en particulier sont connus pour manipuler le cycle cellulaire dans les cellules hôtes infectées (40). Ces observations nous ont suggéré que HHV-6 peut également affecter la progression du cycle cellulaire dans les cellules infectées. Cependant, peu de choses sont connues concernant cet aspect de la biologie virale, surtout à l'initiation de ce projet de doctorat. Cette question a fait l'objet de recherches présentées dans cette thèse.
Nous avons compilé des informations concernant la biologie de ce virus dans une revue exhaustive. Nous joignons cette revue à la thèse. D'autres détails relatifs à tous les aspects du virus se trouvent dans cette revue.

Revue sur le Virus Herpes Humain-6 HHV-6:

The Human Herpes Virus-6: An Exhaustive Review.

Article 1: Olfa Debbeche, Suzanne Samarani, Alexandre Iannello, and Ali Ahmad

Résumé: HHV-6 est le sixième membre de la famille des herpèsvirus, qui infecte les humains. La plupart des primo-infections par ce virus se produisent à un âge précoce chez les enfants. Les infections deviennent généralement latentes, elles persistent dans différents organes et tissus et font l'objet de faible réplication. Les infections latentes se réactivent souvent dans des conditions d'immunosuppression. Plus de 90% des humains adultes sont infectés par ce virus. Le virus infecte préférentiellement les cellules T CD4 +. Il peut également infecter d'autres types cellulaires comme les monocytes, les macrophages, les cellules microgliales, les oligodendrocytes et les cellules B, mais avec moins d'efficacité. Il est le seul virus herpès humain connu à s'intégrer dans le génome de l'homme et à se transmettre congénitalement.

Le spectre des maladies et des syndromes associés à HHV-6 est de plus en plus large et comprend la roséole, la mononucléose infectieuse, le rejet de greffe, la sclérose en plaques, l'encéphalite, l'épilepsie, le cancer, l'hypersensibilité médicamenteuse, les éruptions cutanées, la progression du sida, etc. Dans cette revue, nous discutons la biologie, l'immunologie et la pathogenèse du virus et les relier à diverses manifestations cliniques des maladies et syndromes causés/associés par ce virus.

The Human Herpes Virus-6: An Exhaustive Review

Olfa Debbeche, Suzanne Samarani, Alexandre Iannello, Ali Ahmad

SUMMARY

HHV-6 is the sixth member of the Herpesvirus family, which infects humans. Most of the primary infections with this virus occur in early age in children. The infections usually become latent, persist in different organs and tissues in the body and undergo low-grade replication. Latent infections frequently become reactivated upon immunosuppression. More than 90% of the adult humans are infected with the virus. The virus preferentially infects CD4+ T cells. It can also infect other cell types like monocytes, macrophages, microglia, oligodendrocytes and B cells, albeit with less efficiency. It is the only human Herpesvirus known to become integrated in human genome and be transmitted congenitally. The spectrum of diseases and syndromes associated with HHV-6 is ever increasing and includes roseola, infectious mononucleosis, graft rejection, multiple sclerosis, encephalitis, epilepsy, malignancy, drug hypersensitivity, skin eruptions, AIDS progression, etc. In this review article, we discuss biology, immunology, and pathogenesis of the virus and relate them to various clinical manifestations of the virus-caused/associated diseases and syndromes.

INTRODUCTION

In 1986, a virus was identified in *in vitro* cultures of the human peripheral blood lymphocytes (PBL) obtained from AIDS patients suffering from lymphoproliferative disorders [1]. The virus caused formation of syncytia and appearance of large refractile balloon-shaped cells in cell cultures. The authors named it as the Human B-lymphotropic virus (HBLV) as they had observed it in the PBMC of the patients with B cell lymphomas. However, in later studies, it was shown to be a Herpesvirus and was renamed as the Human Herpesvirus HHV-6, since at that time it represented sixth Herpesvirus that could infect humans. Initially the virus was placed in the γ-Herpesvirinae subfamily along with the Epstein-Barr virus (EBV). Realizing that HHV-6 resembled more closely with the Human Cytomegalovirus (HCMV) than HSV-1 or EBV, it was classified as a member of the β-Herpesvirinae subfamily [2]. Since its discovery, a lot has been learnt about the virus, its replication, pathogenesis and clinical manifestations, and several excellent reviewes have been written[3-7]. However, these reviews deal either with certain specialized aspects of the virus and its infection or need updates. Therefore, we decided to write an exhaustive review covering all aspects of the virus, its biology, host response, pathogenesis, diseases and clinical syndromes.

The family Herpesviridae includes all Herpesviruses. The word 'Herpes' means crawling; a name given to the insidious nature of the infections caused by these viruses. These viruses tend to be species-specific. Each species has a set of its own Herpesviruses. A common characteristic of these viruses is their ability to cause latent or low-grade persistent infections in their natural hosts and reactivate under conditions of immunosuppression. To date eight Herpesviruses are known to infect humans. Based upon the genetic analysis of the conserved structural glycoproteins, they are divided into three subfamilies named α-, β- and γ-Herpesvirinae (Table 1). The α-Herpesviruses are neurotropic, β- have more confined cell tropism and γ- are lymphotropic with oncogenic potential. Herpesviruses are widely prevalent in human populations with the exception of HHV-8 and HSV-2.

Overtime many HHV-6 viruses have been isolated from humans from different parts of the World. It was realized that these HHV-6 islolates could be divided into two types, A and B. This classification was based upon several characteristics: the reactivity of viral proteins with monoclonal antibodies, tissue tropism, epidemiology, sensitivity to antiviral agents, restriction fragment length polymorphism (RFLP) of the viral DNA, association with distinct disease patterns, etc [8-11]; see Table 2 for different characteristics of A and B variants). The A variants represented by the viral strain GS are relatively more neurotropic and infect HSB-2 and Sup T1 cells but not Molt-4. Their DNA is more frequently detected in CSF. The B variants represented by the strain Z-29 infect Molt-4 but not HSB-2 or Sup T1. Furthermore, they preferentially establish infections in salivary glands, where they undergo persistent low-grade replication in epithelial cells and are frequently found in saliva and blood ([10]; reviewed in [12]). About 99% of primary HHV-6 infections in children are caused by the B variants [13]. However, in subsaharan HIV-

endemic areas, children are often infected with A variants [14]. Interestingly, to date no recombinant viruses between A and B variants have been described. See Table 3 for differences in A and B variant with respect to their sites of recovery in different disease conditions. Suggestions have made to upgrade A and B variants to distinct viral species.

HHV-6 resembles with HHV-7 very closely in DNA sequence and antigenic characteristics. Together, the two viruses form a unique genus called Roseolavirus. HHV-7 shows little changes in individual isolates, and has no evidence of division into different types or variants (reviewed in [12]).

CHARACTERISTICS OF HHV-6

Like all Herpesviruses HHV-6 is an enveloped virus with 160-200 nm diameters [15,16]. A typical structure of the virion is shown in Figure 1. It consists of three major structural elements: nucleocapsid, tegument and envelope. The nucleocapsid is composed of a torus-shaped core surrounded by a capsid. The core consists of a single linear double stranded DNA molecule of about 160-170 kb and associated proteins. The tegument occupies the space between core and envelope. It is amorphous, sometimes asymmetrical and contains many viral proteins and enzymes. In HCMV, which is very closely related to HHV-6, tegument contains more than thirty-five proteins. Upon viral entry, some of them detach and mediate transcription of Immediate Early (IE; see below) genes. Others mediate movement of the nucleocapsid along microtubules to the nuclear pore. Certain tegument proteins also move newly formed capsids inside nucleus via infection

n-induced actin, mediate budding of the nucleocapsids into inner nuclear membrane and are needed for viral assembly and immune evasion [17,18]. The envelope is the outermost structure of the virion and is essentially a lipid bilayer. It is derived from the patches of the plasma membrane of the infected cell into which a dozen or more distinct viral glycoproteins have been inserted. The viral glycoproteins are embedded as short spikes in the lipid bilayer of the envelope. A single virion may contain as many as one thousand or more copies of each glycoprotein (reviewed in [19,20]).

MOLECULAR BIOLOGY

HHV-6 has a linear double-stranded DNA molecule with an average length of 160-170 kb. The genomes of three viral strains, an A type variant (U1102) and two B type variants (Z29, HST), have been fully sequenced [21,22]. Each genome consists of a central unique (U) region of 143-144 kb flanked by a 8-9 kb region of terminal direct repeats (DR) on either side (shown schematically in Figure 2). The U regions of the two viruses, HHV6 and HHV-7, resemble with each other co-linearly and are equivalent to the unique long (UL) region of HCMV. The HHV-6 U region is interrupted by the presence of three repeats (R1-R3) near the right end in the Immediate Early (IE)-A region. The ORFs encoded in the DR and U regions are prefixed DR and U, respectively and are numbered

from left to the right. The U and DR regions contain 100 and 7 open reading frames designated as U1-100 and DR1-7, respectively. DR regions also contain motifs for cleavage and packaging called pac1 and pac2. Each DR contains two human telomerase-like repeat sequences; one at its end and the other at its junction with the U region. These repeat sequences are hexanucleotide GGGTTA and play a role in DNA replication and maintenance of viral genomes in latently infected cells [23,24]. There are seven blocks of genes, which are conserved in all herpesviruses and are located in the center of the viral genome. These genes encode viral structural proteins and enzymes needed for nucleotide metabolism and viral replication. Another group of genes (U2-U19) is located to the left of the seven-block region. The group contains genes that are equivalent to the US-22 gene family of HCMV. The US-22 gene family is unique to the members of the β-Herpesvirinae subfamily. Most, if not all, members of gene family encode tegument proteins, and may play a role in viral replication immediately after penetration [18,25]. Eleven members of family (DR1, 2, 6, 7, U7, 8, 16, 25 and 95) are present in HHV-6. Some of them are expressed as IE proteins and are likely to act as transcriptional activators.

Based upon their temporal sequence of expression in the course of replication, Herpesviral genes are classified as Immediate Early (IE or α), Early (E or β) and Late (L or γ). The IE genes are expressed immediately after infection in the presence of inhibitors of protein synthesis. The HHV-6 IE genes occur in two blocks IE-A (U90/U86-89) and IE-B (U16-19). They are very divergent and may also differ in their splicing pattern and temporal expression between A and B variants [26]; See Table 4 for divergent genes in HHV-6 variants and other Herpesviruses). The expression of IE genes is controlled by the intermediate repeat region R3, which contains DNA sequences for binding cellular transcription factors e.g., NF-kB, AP-2, the Polyoma enhancer activator (PEA)-3, etc. The region contains more NF-kB sites in A variants. The transcription may start from multiple promoters depending upon the stage of infection resulting in multiple transcripts of different sizes [27,28]. Other genes expressed with IE kinetics include U42, U94, U73 and U39, etc (see Table 5 for important HHV-6 genes, their encoded products and functions). The IE gene encoded proteins mainly serve regulatory functions and are essential for expression of E genes.

E genes can be expressed in the presence of inhibitors of the viral DNA polymerase but not in the presence of inhibitors of protein synthesis. They play a role in DNA replication. Their expression requires de novo protein synthesis in the infected cells. They include U27, U38, and U43/73/77.

Many of the IE and E genes encode proteins that act as transactivators, i.e., they bind *in trsans* to DNA elements in the promoter regions of other viral and/or cellular genes directly or indirectly and induce their expression. These viral transactivators include the followings:

1. PU3: As the name implies, it is encoded by the U3 gene, which is a positional homologue of the HCMV UL24 gene. PU3 can regulate gene expression. It represents one of several tegument proteins that are present

in the virions of the beta-Herpesviruses (reviewed in [18]). It has been reported to activate HIV LTR in monkey kidney cell line CV-1 [29].

2. Direct Repeat (DR)7: It is expressed within 18 hours postinfection with Early/Late (β-2) kinetics. Encoded by Orf-1 present in the Sal-1 L fragment of the HHV-6 genome, DR7 protein binds and inactivates the tumor suppressor p53 and has oncogenic potential. The protein also promotes HIV LTR activity in monkey fibroblasts and human T cells. The protein transformed NIH3T3 cells, which formed fibrosarcoma in nude mice ([30,31]; reviewed in [32]. From intact genome, Orf-1 encodes a DR7 starting from within DR6 [33]. Interestingly in B variants the DR7 homologous protein localizes to the nucleus with the viral DNA Polymerase Processivity Factor p41, and not with p53 [33].
3. U27: It encodes p41, the DNA polymerase processivity factor [34,35]. It transactivates HIV LTR in CV-1 cells. This transactivation depended upon the presence of NF-κB binding sites in the promoter [36]. Its homologue pUL42 binds and activates CDK1 in place of cyclin B in HSV-1 infected cells [37].
4. U94: It is one of the unique genes that are present only in HHV-6. It is an analogue of the human Adeno-associated parvovirus virus-2 *rep68/78* gene [38]. The AAV-2 Rep proteins are multifunctional, have sequence-specific single stranded DNA binding, nicking and helicase activities. These non-structural proteins mediate site-specific integration of AAV-2 into human chromosome 19q13.3. The gene is also essential for the replication of the virus. Three and $2x10^5$ Rep-binding sites exist in the viral and human genomes, respectively [39]. It has been shown to inhibit several heterologous promoters including that of HBV and HCV [40]. U94 is highly conserved in both A and B variants of the virus. Its product, a 490 amino acid polypeptide, is a transcriptional repressor and can regulate several autologous and heterologous promoters. U94 is expressed with IE kinetics in latently infected cells. However, the protein can also be detected in small amouts in productively infected cells. Its product inhibits HHV-6 replication and plays a role in maintaining viral latency. It localizes to nucleus and binds the TATA-binding protein and also has the ability to bind ssDNA [38,41-43]. U94 protein can inhibit gene expression from HIV-LTR-CAT constructs in a TAR-dependent manner. The protein also acts as a tumor suppressor and inhibits H-ras-mediated cell transformation ([44,45]; Reviewed in [4]). U94-expressing cells can be infected with HHV-6, but the virus does not replicate in these cells. The expression of this gene does not seem to cause any cytopathic effect in human cells [42]. The protein also inhibits replication of HBV and beta-Herpesviruses in human cells but not of HSV-1 [46]. When expressed in vascular or lymphatic endothelial cells, it exerts anti-angiogenic effects and inhibits these cells to form capillary-like structures [47].
5. IE-A Proteins: The HHV-6 IE-A locus expresses many splice variants and is supressed by the CpG methylation. The locus encodes two proteins: IE1 and IE2, which correspond to orf U90/89 and U90-86/87, respectively. Each contains an exon derived from the U90 gene [48,49]. Temporally IE-1 is expressed earlier than IE-2. The latter protein binds IE-1 promoter and may inhibit its transcription. This may explain a transient expression of IE-1 but continuous increase in the expression of

IE-2 in HHV-6-infected cells [50]. IE-1 can transactivate heterologous promoters including HIV LTR [51,52]. The protein from HHV-6 B variants has less transactivating potential compared to the one from A variants [27]. IE1 is phosphorylated on serine/threonine residues, sumoylated and is localized to nucleus along with promyelocytic leukemia (PML) proteins [27]. IE2 has transactivating potential for complex and very simple (that may contain only one or no response element) promoters. It appears that IE-2 plays a role in the transactivation of both viral and cellular promoters. It was shown to induce expression of CD4 and COX-2 genes in human cells [27,53,54]. Unlike IE-1, IE-2 is not sumoylated. It interacts with the ubiquitin-conjugating enzyme 9 (Ubc9), beta subunit of casein kinase II and heterogeneous nuclear riboprotein K (hnRPK) in the yeast two-hybrid system [55,56]. Ubc9 is involved in the SUMO conjugation pathway. Its over expression represses transactivational potential of IE-1. The hnRPK is implicated in preRNA processing. Some conflicting reports exist as to the temporal expression of some U90//U86-87 transcripts as IE time points [27,28,49,57].

6. U95: The HHV-6 gene U95 is a member of the HCMV US22 gene family. Its positional homologue in MCMV encodes IE-2. U95 is located upstream R3 and is expressed as an IE protein [58], which is likely to have transactivating potential. Recently it was shown that the U95 IE protein from B variants interacts with GRIM-19: a gene belonging to the family of genes involved in retinoic acid and interferon-induced mortality. GRIM-19 associates with a serine protease HtrA2 and mediates cell death [59]. GRIM-19 inhibits HtrA2-mediated destruction of XIAP. It also inhibits activation of STAT-3. *In vitro*, siRNA against U95 reduces viral replication, cytopathic effect and mitochondrial permeability potential [60]. GRIM-19 is involved in the mitochondrial oxidative phosphorylation system. U95 interaction with GRIM-19 is responsible for HHV-6 effects on mitochondria, i.e., cell death by expiration rather than apoptosis.
7. IE-B Proteins: The U16-19 genes of the HHV-6 have similarities with the UL36-38 genes of HCMV. The genes belong to the HCMV US22 gene family, which encode proteins with transactivating potential for viral and cellular promoters. The genes in the locus undergo multiple splicing and are temporally regulated. U16/17, U18 and U19 encoded IE-B proteins have transactivating properties and each can transactivate HIV LTR *in vitro* [61-64]. Some U16/17 transcripts may appear later in B variants [26].
8. Other transactivators expressed by HHV-6 include U42, U51 and U54. All Herpesviruses express U42 transactivator. U51 is a novel chemokine receptor that binds RANTES and causes its downregulation by a novel mechanism [65]. U54 is a homologue of the HCMV tegument protein pUL42, which enhances transcription from the promoters containing AP-1 and ATF cis elements [66].

The late genes include U39, U82, U48, U18, U97-100, etc. They encode viral proteins necessary for assembly and release of the virions. The late gene-encoded glycoproteins are implicated in the infection process (attachment to the target cells and penetration). Of these genes, U39 encodes gB, which plays a role in virus attachment and penetration. Interestingly, despite being a late gene in other Herpesviruses, it is expressed as IE gene in HHV-6 (Table 5; [26]). It is one of the proteins,

which are present in the primary envelope of the viral capsids as they bud into perinuclear space. The glycoprotein is conserved in all Herpesviruses. The HHV-6 gB shows 39% sequence homology at amino acid level with that of HCMV, leading to immunological cross reactivity between the two viruses. It is produced as a precursor molecule, which is proteolytically processed into two functional sub units of 64 kD and 58 kD in A variants (e.g., U1102) and into three subunits of 102 kD, 59 kD and 50 kD in B variants [67].

The late gene U82 encodes a glycoprotein, gL, which forms a covalent complex with the glycoprotein gH, which is encoded by U48. The gH-gL complex is involved in the infection and fusion process of the virus. The gL plays a role in the transport and processing of the complex in all β-Herpesviruses [68]. The gene U100 or Q encodes gpQ, which is present only in the Roseaolavirus members. The gene shows only 72% sequence identity between the two HHV-6 variants. Hence, it may play variant specific roles in the infection process. In HHV-6A, the Q gene (U100) encodes two splice variant products: an 80 kD Q1 with 6 N-linked glycosylation sites and a 37 kD Q2 with 4 N-liked glycosylation sites. The two Qs form tetrameric complexes with the gH-gL dimer within the endoplasmic reticulum. The complexes are expressed on the surface of the viral envelope (reviewed in [19]). The exact stochiometry of the complexes remains unknown. The gH-L-Q1-Q2 complex from A variants can efficiently bind human CD46 but the complex from B variants differs in its ability to bind CD46. The complex from PL and Z29 strains binds but not the one from HST. The 80 kD Q1 component of the complex specifically binds the viral receptor, CD46 [69-71]. U18 encodes a multiply spliced hydrophobic virion glycoprotein, gM, whose exact function remains unknown [72]. U47 is a positional homologue of the HCMV gpO gene. U47 bears 76.8% sequence identity between the two variants of HHV-6. GpO forms a third component of the gH-gL complex. The gH-gL-gO complex does not bind to CD46 [73]. The complex may bind to an unknown viral receptor on human cells.

The envelope glycoproteins gB, gH and gQ contain epitopes for virus neutralization.

U11 gene encodes a phosphoantigen p100, which is the major antigenic structural protein of HHV-6. The protein has only 80.1% amino acid sequence homology between A and B variants. The U53 gene encodes a viral protease, which is auto-cleaved at two sites and is necessary for viral assembly and maturation.

A protein kinase encoded by U69 imparts sensitivity to gancyclovir ([74]; reviewed by [75])**.** Mutations in U69 may lead to ganciclovir-resistant viruses (see below in the section on Drug Resistance). Its homologous protein from HCMV pUL97 has also been shown to phosphorylate pRB and induce cell cycle progression in the virus-infected cells [76]. The hyperphosphorylated pRb also inhibits formation of aggresomes, which contain PML and viral IE proteins [77]. The aggresomes sequester viral proteins and prevent viral replication.

It is noteworthy that products of several ORF in HHV-6A are translated into proteins, which are shorter than their HHV-6B counterparts.

Several genes are found exclusively in HHV-6 and HHV-7 and not in any other herpesvirus. They include U20-24, U24A, U26, U85 and U100.

The A variants have 110 ORF whereas B variants have 119 ORF. Nine of the B variant genes (designated B1-B9) have no counterparts in A variants. The 119 orf in B variants compose 97 unique genes [22]. Furthermore there are 9 orfs described in A variants that have no counterparts in B variants [23]. The members of the two different variant types show 90% identity at nucleotide level. The central conserved region has 95-98 % identity and decreases as one reaches the genome ends. It decreases to 30% in the some of the IE-A region genes (U86 to U95; with the exception of U94) [21,22] . The patterns of splicing and temporal expression of several genes also differ between the two variants (see Table 4). In addition to IE-A, the two variants differ in DNA sequence in U97-U100 gene region. This region encodes the gp82-gp105 complex. This region may be responsible for different biological characteristics of the two variants. One exon of this region HN3 is found only in HHV-6 B variants [21]. The transcripts from this region undergo extensive splicing and produce variant specific neutralizing epitopes. Differential variant-specific splicing of the mRNA from these genes results in variant specific proteins and neutralizing epitopes recognized by variant specific antibodies [71,78].

Other regions, which differ between the two variants, occur in LT-1, DR2, DR7, DR8, LJ1, RJ, U1 and U47 [21]. Among these genes, only the functions of DR7 are known. Several genes (e.g., U94 and U83) are unique to HHV-6.

The A and B variants of HHV-6 show 94% identity at the amino acid level in the seven-region conserved block. They differ from each other in the DR region as well as in a 24 kb segment to the right of U85 excluding U94. The latter gene differs only by 2.4%. These genetic differences between the two variants translate into biological differences between the two variants [79].

As mentioned above, the DNA sequence of the IE-A region varies between A and B variants. However the sequence remains highly conserved within isolates of A variants. The IE-A region sequences in B variants tend to segregate into two subgroups represented by Z29 and HST strains [80]. Furthermore, the sequences in the left ends of the two DR regions may also vary in different isolates of the same type ([23,24]; reviewed in [4,81]).

HOST GENE PIRACY

The Herpesviruses are expert in pirating host genes. Like other herpes viruses, HHV-6 has usurped several host genes, e.g., U83, U12, U51, and DR7 (see Table 6 for the virus-pirated genes). U83 encodes a protein with beta chemokine-like properties called a virokine. It is expressed an early gene present in both A and B variants and is a hot spot for DNA variation [82]. The U83 proteins encoded by the two variants differ significantly from each other. The gene from A variants undergoes alterate splicing using very unconventional splice sequences and produces a full-length (10.4 kD) and a more abundant N-truncated

shorter (5.5 kD) protein [83]. The A variants encode proteins without a signal peptide at its N terminus and hence are not secreted from the cells [84]. The full-length protein, U83A, can bind several human C-C chemokine receptors (CCR1, 4, 5, 6 and 8) and is expressed late in infection. It induces chempattraction of TH-2 cells, which express CCR4 and CCR8. The virokine shows higher affinities for CCR1 and CCR5. Its splice variant N-truncated (U83A-N) is expressed early in the course of the infection and acts an antagonist for CCR1 and CCR5 [85]. It is noteworthy that CCR1 and CCR5 are expressed on the surface of monocytes, macrophages and dendritic cells. The full-length virokine attracts these target cells and promotes viral spreading. It has been shown that U83A could effectively prevent infection of human cells by M-tropic HIV strains via CCR5 and U83N also does so but less efficiently [86]. Importantly, the truncated virokine is secreted early in the course of the infection to prevent the influx of inflammatory cells to the site of infection [84,85,87].

The U83 protein encoded by B variants, U83B is a functional homologue of human CCL-4. It binds only CCR-2 and acts on lymphocyte and monocytes. The protein can induce Ca^{++} mobilization and chemotaxis in CCR2-positive THP-1 cells, monocytes and macrophages [84,87]. The virokine is not differentially spliced and is expressed as a late gene.

U22 encodes another virokine unique to HHV-6. Not much is known about it.

U12 and U51 genes encode viral proteins, which can act as homologues for different G-Protein-Coupled Receptors (GPCR). By their ability to bind certain C-C chemokines, U12 and U51 proteins act as soluble chemokine receptors or viroreceptors [23]. These decoy receptors neutralize host chemokines and modulate immune response to the advantage of the virus. The U51 protein can also promote viral fusion and syncytia formation. U51A also promotes viral replication by inducing fusion and cell-to cell spread of the virus [88]. Inhibiting the expression of this gene by siRNA reduces the virus-induced cytopathic effects in *in vitro* cell cultures [88]. When expressed on epithelial cells it binds the chemokines CCL2, 5, 11, 7 and 13 [65,89]. U51 is expressed early in infection on the surface of T cells [90]. In other cell types like human epithelial kidney cells and osteosarcoma cell lines, the protein accumulates in the endoplasmic reticulum. The protein binds RANTES and inhibits its secretion [65]. The mechanism of action involves transcriptional downregulation and morphological changes [65]. U51 exhibits some constitutive activity, as it can activate phospholipase C and inhibits cAMP-mediated gene transcription [91]. U51A responds to CCL2, 5, 7, 11 and13 by calcium influx. This specificity overlaps with that of CCR1, 2, 3 and 5. However, it also acts as a decoy for XCR1 and CCR7, which are expressed on human NK cels, DC and T cells [89].

The U12-encoded protein is related to CCR-1, 3 and 5. It is expressed at a late stage of infection in CBMC and macrophages and can neutralize β-chemokines like RANTES, MIP-1α, MIP-1β, MCP-1 but not α-chemokines [92,93].

VIRAL ENTRY AND VIRAL RECEPTOR

It is believed that the viral glycoprotein gB (pU39) initiates the infection process. It binds to heparan sulfate proteoglycans (HSPG) present on the surface of the target cells and thus enables the virus to attach to its specific receptor, CD46. It may also play a role in membrane fusion. The role of gB, however, may not be essential, since heparin, which inhibits the interaction between HSPG and the gB protein does not inhibit HHV-6 infection. However neutralizing epitopes have been described on this protein [94]. It is noteworthy that gB is highly conserved in all Herpesviruses. The gB from HSV-1 has been shown to bind the Paired Immunoglobulin-like Receptor (PILR)-α, which is an inhibitory receptor specific for binding with CD99, and is expressed on the surface of CD14+ monocytic cells [95]. Whether gB from HHV-6 binds PILR-α remains unknown.

CD46 has been convincingly demonstrated to act as a receptor for HHV-6 [96,97]. B variants may bind less efficiently or not at all with CD46. In Chinese Hamster Ovary (CHO) cells transfected with human CD46, A, but not B, variants can enter in the cells. The molecule is downregulated on the surface of the infected cells. Anti-CD46 antibodies block infection with both A and some B variants. Soluble CD46 blocks the envelope-mediated fusion. Expression of CD46 in non-human cells makes them susceptible to the viral entry and fusion [97]. However its expression alone is not sufficient for viral fusion and infection suggesting the requirement for additional cellular molecules (co-receptors) for infection. It was demonstrated that the chemokine receptors CXCR4 and CCR5 do not play a role in HHV-6 infection of target cells ([98]; reviewed in [32]). It is noteworthy that these two receptors act as essential co-receptors for T-tropic and M-tropic HIV-1 strains, respectively. Furthermore, it has also been demonstrated that although HHV-6 preferentially infects human $CD4^+$T cells, it does not use CD4 as a viral receptor or co-receptor.

CD46 belongs to the family of membrane-bound complement regulatory proteins (mCRP). The other members of the family include CD55, CD59 and Complement Receptor type 1 (CR1). The mCRP regulate complement activation events on the host cell surface. CD 46 has also been named as “membrane co-factor protein”. It is a type 1 transmembrane glycoprotein, which was first described to regulate the cascade of complement activation and induction of immune responses. CD46 occurs on almost all nucleated cells in the body including microglia, astrocytes and oligodendrocytes [99]. It could also act as a co-stimulatory molecule for T cells [100].

CD46 binds C3b and C4b and allows their inactivation via Factor 1 and inhibits their conversion into chemoattractive convertases. It consists of four short consensus repeats (SCR), a serine threonine-rich domain and a sequence of unknown significance in its extracellular region (Figure 3; reviewed in [101]). The extracellular region is followed by a transmembrane (TM) region and a cytoplasmic region (CYT). The extracellular region has several O and N-liked glycosylation sites. The molecule exists in several isoforms, which result from alternate splicing and may vary in

their serine threonine-rich domains, TM and CYT regions. The isoforms may also differ in tissue distribution. The viral tetrameric complex gH-gL-gQ1-gQ2 binds to the CD46 receptor [70]. More specifically, the gH-gL complex binds to the second and third SCR of CD46 [20]. gH also interacts with scr2 and 3 (and 4) of CD46 [96]. A trimeric gH-gL-gO complex has also been identified that does not bind to CD46 (Figure 3). It is noteworthy that CD46 also acts as a receptor for vaccine strains of measles virus. However, the two viruses bind to different domains of CD46 [102]. In addition to measles, B adenoviruses except type 3 and 7, Streptococcus pyogenes, Neisseria gonorrhoea and Neisseria meningitides also bind to human cells via CD46 [103], In animals, bovine viral diarrhea and canine distemper virus also use CD46 as a receptor for infecting target host cells [104].

The penetration of HHV-6 in the infected cells is mediated by endocytosis and is pH sensitive. It can be inhibited by antibodies to the viral glycoprotein complex gp100, gB, gH, gQ or CD46 [69,105,106].

It is noteworthy that both A and B variants vary in their ability to bind CD46 and induce cell fusion from without in CD46-positive human cells. At least some B variants are unable to induce this fusion [99,107]. The fusion occurs within one hour without virus replication or protein synthesis. Antibodies to gB, gH and CD46 can block this fusion. The process may contribute towards the pathogenesis of the infection.

CELL AND TISSUE TROPISM:

Both A and B variants can efficiently infect activated peripheral blood and cord blood mononuclear cells. The virus preferentially infects activated CD4+ T lymphocytes. Both CD25+ and CD25- CD4+ T cells are equally infectable with the virus [108,109]. These cells serve as primary targets for viral replication as well as reservoir for latent infection. A, but not B, variants can also efficiently infect CD8+ T cells [110]. HHV-6 can also infect various other cell types, eg., monocytes, macrophages [111], NK cells [112], fibroblasts [113], cervical epithelial cells [114], megakaryocytes, Burkitt's lymphoma cells and EBV-transformed B cells [115,116]. These cells, however, are not infectable as efficiently as CD4+ T cells. Both A and B strains can infect dendritic cells (DC). In this regard, one group did not see signs of productive infection in *in vitro* monocyte-derived dendritic cells [117]. However, another group detected late viral antigens in the virus-infected cells but no cytopathic effects [118]. More recently, it was shown that nucleocapsids develop in these cells but do not mature. These virus-infected cells can, however, efficiently transmit virus to CD4+ T cells [119]. In this regard both myeloid and plasmacytoid DC obtained from peripheral blood were shown to efficiently transmit the virus to T cells and shed it in the culture medium.

The virus can infect early CD34+ hematopoietic stem cells latently. Upon differentiation, the infection becomes transmitted longitudinally to different cells types including monocytes, macrophages, dendritic cells, etc. [120]. The virus can also infect primary human astrocytes as well as human progenitor-derived astrocytes productively. In this regard A variants, but not B ones, infected astrocytes productively without

inducing annulate lamellae [121]. B variants of the virus mediated cell-cell fusion in cultured microglia, astrocytes and oligodendroglia [122]. The virus infected the cells but did not multiply. However, the virus did not infect NT2D, a fully differentiated neuronal cell line. The virus infected and induced death in oligodendrocytes that could not be prevented with caspase inhibitors [123]. Glial cells, which represent precursors of oligodendrocytes, were also shown to be productively infectable with the virus [124]. The infected cells do not differentiate into mature myelin producing oligodendrocytes.

Interestingly, human vascular and lymphatic endothelial cells were shown to be productively infected with the virus without showing cytopathic effects [125]. These cells express high levels of U94 indicating that the infection remains latent.

With respect to the infection of human monocyte-derived macrophages (MDM), researchers reached different conclusions: no infection [126], transient infection [111] and full viral replication with cytopathic effects [127]. It was also reported that monocytes were resistant but their differentiation with IL-15 made them susceptible to infection with this virus [128]. These studies suggest that the cells are infectable under appropriate conditions. In acute HHV6 infection, more viral genomes were detected in monocyte macrophages as compared to CD4+ T cells. The infected monocyte macrophages expressed U79/80 mRNA indicating active viral replication. This suggests that the viral replication in these cells contributes to the viremia that is seen in acute infections [92]. The two cell types (monocyte-macrophages and vascular endothelial cells represent sites of viral latency in humans).

It has been found that *in vitro* type A and B isolates replicate most efficiently in HSB-2 and MOLT-3 cells, respectively. The virus usually takes 8-12 days to fully replicate and produce infectious virions in these cells. HSB2 are the most permissive cells for A variants. About 90% of these cells become infected by day 9 postinfection compared to HEL, a megakaryocytic cell line, of which only 2% cells are infected on day 9 [129]. The virus may complete its replicative cycle in primary lymphocytes in 5-7 days [130].

HOST SPECIES

The human Herpesviruses are usually very species specific. However, HHV-6 was found to infect and cause cytopathic effects in the chimpanzee PBL cultures [131]. Both A and B variants were also able to infect PBL from Pigtailed macaques (Macaca emestrina); however only A variants infected these cells from Rhesus (Macaca mulatta) monkeys [132]. In vivo, co-infection with HHV-6A accelerated AIDS progression in Pig-tailed macaques [133,134]. In the co-infected animals, SIV evolved to resist RANTES. Serological studies have suggested that simians might become naturally infected with HHV-6 or with a closely related virus [135,136]. In fact, Lacoste et al. isolated an Herpesvirus belonging to the Roseolavirus from wild monkeys and mandrills, and named it as Mandrill Herpesvirus (ManHV; [137]. The same group identified another HHV-6-like virus from chimpanzees and named it as PanHV-6 [138]).

ANIMAL MODELS:

HHV-6 does not infect mice. Gobi et al developed a mouse model of the infection [139]. The group transplanted human thymus and liver in SCID mice and showed that a type A strain of HHV-6 (GS) could efficiently infect the transplanted thymus in the SCID-hu Thy/Liv mice [139]. The infection caused a progressive and severe depletion of human thymocytes in the mice affecting all major cell types. These results suggest that HHV-6 may cause similar depletion of thymocytes in humans. As stated above, chimpanzees and Rhesus monkeys can be infected experimentally with the virus. However, use of these animals for research is impracticable due to high cost and ethical considerations.

EPIDEMIOLOGY AND TRANSMISSION:

HHV-6 appears to be a ubiquitous virus. Serological studies conducted in different parts of the world show that about 90% of the children are infected with this virus early in their childhood [140,141]. Most of the newborn babies are seropositive due to the presence of their maternally derived antibodies, however these antibodies disappear pregressively and by the age of six months, the children become seronegative [142]. These antibodies protect newborns from early infections. Most of the infections with the virus in children occur at 6-9 months of age in North America [143]. Epidemiological data have shown that by the age of 12 months, 50-60% and by the age of 2-3 years, about 90% of the children become infected with the virus [13,140,141]. The children appear to acquire the infection via saliva of their parents, adult siblings, family elders or adult caregivers. It was demonstrated that the virus isolated from the blood of children suffering from roseola (primary infection) resembles with the virus obtained from the saliva of their mothers suggesting intra-family horizontal transmission of the virus via saliva [144]. The saliva sharing habits of the family may affect the age at which children may become infected. Having older siblings and parents who shared saliva frequently increases the risk of HHV-6B infection earlier [145].

The viral DNA could be detected in the cordblood lymphocytes (CBL) in healthy newborns in the absence of anti-viral IgM in serum as well as from spontaneously aborted fetuses. HHV-6 becomes reactivated frequently in mothers but the infection is transmitted vertically to newborn babies ony in 1-2 % of the cases. The incidence of vertical transmission occurs independently from HCMV transmission [146-148]. In one study, about 20% and 3% of the pregnant women carried DNA sequences from HHV-6B and HHV-7 respectively in their genital tracts but perinatal transmission remained rare [149]. Acutely infected children secrete virus persistently or intermittently in saliva and stools but not in urine [150,151]. Despite this, infections via feco-oral route have not been reported. Similarly, there is no evidence of the viral transmission through sexual activities.

The congenital infections that occur in 1-2% newborn babies result from integration of viral genome in the genome of the mother or father, and its

transmission to the offspring via gametes [152]. The children with chromosomally integrated viral genome usually have high viral loads in all specimens and produce virus-specific antibodies. The integrated viral genomes were predominantly from A variants [153].

In the hospital setting, nosocomial infections usually occur with blood transfusions, organ and bone marrow transplantations ([154,155]; reviewed in [156]). The infection to the seronagative recipient could occur due to infected transplanted organ, blood or progenitor stem cells. In some rare cases, the infections may occur from cord blood transplantations as the donor may have HHV-6 integrated in his/her genome.

The incubation period of the infection is usually of 2-3 weeks duration. The children usually recover from the acute primary infections, however they are never able to get rid of the virus and become life-long carriers of the virus. The virus becomes latent and localizes itself in salivary glands. These glands represent one of the major reservoirs of HHV-6 infection in humans. The virus undergoes continuous or intermittent low-grade replication in these tissues. The viral proteins and DNA sequences can be easily detected in salivary tissues of HHV-6-infected persons. The virus is shed persistently or intermittently in the saliva of HHV-6-seropositive individuals [157]. All the virus isolates obtained from saliva are of B variants. The virus could also persist in monocytes in the peripheral blood, lungs and brain.

The primary infections in children occur mostly with B variants [13]. It is noteworthy that these variants could be isolated frequently from the peripheral blood lymphocytes (PBL) of the healthy children having primary HHV-6 infections. On the other hand, A variants can be rarely isolated from the PBL of these children. The A variants have rarely been isolated alone independently of B variants from immunocompetent children having Roseola or other febrile illnesses. However these variants have been more frequently isolated from cerebrospinal fluid (CSF) than from saliva or PBL. From the patients infected with both A and B variants, one may isolate A or B variants from CSF, but only B variants can be isolated from the PBL. Similarly from young children with clinical symptoms of fever and sepsis, A but not B variants were isolated from their CSF [158]. A variants could also be isolated relatively more frequently from lungs and skin. These studies clearly show a differential tropism of A and B variants for different tissues of the body: A variants favoring brain, lungs and skin, and B variants for blood and salivary glands.

The virus has worldwide distribution. The prevalence rate may vary in different geographic locations from 75-100%. In developed countries, more than 95% of the adult population is seropositive for one or both variants of HHV-6. The persons with higher antiviral antibody titers (>1280) respond to both A and B variant antigens, but the ones with lower titers (≤320) respond more often to B than A variant antigens [159]. The antibody titers tend to decline with advancing age (reviewed in [6,12]).

PATHOGENESIS

As stated above, the virus can infect CD4+ T cells and other immune cells and compromises their functions in a variety of ways. The A variants of the virus infect and induce apoptosis in naïve and central memory T cells. They also induce proliferation in effector T cells [160]. The virus inhibits IL-2- and mitogen-induced T cell proliferation, and downregulates expression of CD2, CD3, CD46, CD11a and CD44 in these cells [108,110,161]. It has been demonstrated that the viral U24 protein inhibits the function of a cellular protein TCR40/Asna-1, which is involved in posttranslational membrane insertion pathway, and interferes with recycling of early endosomes. The viral protein prevents recycling of the CD3 chains and targets them to degradation. Consequently, the expression of CD3 on the cell surface is inhibited [162]. Without CD3 expression, the cells cannot perform their normal functions. The viral protein also interferes with the expression of transferrin receptor on the cell surface [163]. The A variants of the virus infects CD4-negative T cells (e.g., CD8+ T cells) and induces expression of CD4+ in them. The co-expression of CD4 and CD8 in the same cells may abrogate their functional abilities. Early viral proteins activate CD4 promoter and induce its expression [52]. The death or malfunctioning of virus-infected T cells compromises ability of the host to induce an effective immune response.

As the virus uses CD46 as a receptor, the expression of this molecule is reduced on the surface of virus-infected cells as well as on non infected bystander cells [97,110]. The virus binds the same parts of the CD46, which are used by the complement. This impairs the ability of CD46 to inactivate complement. This makes immune cells more susceptible to complement-mediated killing in the infected persons. However, a premature killing of the virus-infected cells is prevented by upregulating CD59 and other membrane regulatory proteins, which increase resistance of the cells to complement-mediated killing [164-166]. As mentioned above, HHV-6 (especially A variants) can cause fusion of CD46+ cells without infecting them [107]. It is noteworthy that almost all human cells carry CD46 on their surface [103]. By causing fusion and death of CD46+ T cells, the virus contributes towards pathogenesis of the disease.

As discussed in the above section, the virus can infect dendritic cells. The infection impairs antigen presenting and T cell priming functions of these cells. It reduces the expression of DC-SIGN (CD209) and MHC class I on the surface of these cells [167]. The reduced expression of DC-SIGN on the surface of DC compromises their ability to interact with T cells via ICAM-3.

In immature DC, the virus induces increased expression of both class I and class II antigens but impairs their ability to capture and process antigens [118]. Furthermore, the infection has also been shown to effectively impair differentiation of monocytes into dendritic cells. The DC differentiated from the virus-infected monocytes or generated from the monocytes from patients with reactivated viral infection are defective in antigen presentation and secrete IL-10. Both A and B variants of the

virus were able to interfere with DC maturation in a manner that is independent of the viral replication. The resultant DCs were unable to undergo maturation in response to LPS and IFN-γ as judged by the expression of HLA-DR, CD40, CD80, CCR7, etc. [117,168,169]. Such DCs are unable to prime T cells and mount an antiviral immune response. At least one reason of the dysfunctional DC is their inability to produce appropriate cytokines (see below). The induction of defective DC may play an important role in the pathogenesis of the disease.

The infection of vascular endothelial cells and consequent production of MCP-1 and IL-8 may attract inflammatory cells and initiate inflammatory process [125].

The infection inhibits spontaneous apoptosis in monocytes but decreases expression of CD14, CD64, HLA-DR but not of CD32 [170]. The virus may impair functional activities of these cells. Not surprisingly, HHV-6 was shown to reduce capacity of a human monocytic cell line to kill Cryptococcus neoformans [171].

The virus, especially its A variants, downregulates MHC class I (but not MHC class II, CD1a and CD83) expression on the surface of infected cells [172]. In this regard U21 gene product was shown to bind and divert MHC molecules to endosomal compartments [173]. The A variants are more effective than B ones in this effect. This probably reflects variations in the U21 proteins of the two variants that show 89% sequence identity. Other viral proteins may also be involved in this process. The virus-induced downregulation of MHC class I molecules is preferred strategy of viruses to escape virus-specific CTL responses.

The IE-1 of HHV-6 suppresses IFN-β induction probably by interference with IRF3 dimerization and translocation to the nucleus [174]. Being one of first viral proteins produced in the infected cells, it blocks potential antiviral responses mediated by the host-induced interferons.

HHV-6 can infect brain cells. It was demonstrated in *in vitro* studies that A variants infect oligodendrocytes productively and cause CPE whereas B variants cause latent infection [175]. *In vitro*, the virus has also been shown to infect precursor glial cells [124]. The virus inhibits proliferation in these cells and causes them to arrests at G1/S and ultimate death. The IE gene expression is sufficient to cause these effects. By infecting brain cells, the virus may cause symptoms of the CNS disease.

As stated earlier, primary HHV-6 infections usually become latent and persist in different cells and tissues of the host. The preferential sites of the viral latency and persistence include monocytes-macrophages, vascular endothelial cells, salivary glands, brain, and kidneys. The virus may become reactivated and cause persistent local or generalized infections, especially in immunocompromised individuals.

The cells infected *in vitro* with HHV-6 become 2-5 fold large, balloon-shaped and refractile. The infection arrests cell cycle progression [176]. The cells may ultimately undergo apoptosis, necrosis or a combination of the two. The virus has been shown to cause apoptosis in non-productively infected cells as well as in uninfected bystander cells. The infection may lead to loss of immune cells and immunosuppression. The HHV-6-

induced immunosuppression could potentially promote the pathogenesis of other viral infections, e.g., HIV-1, HCV, and HCMV (see below).

The virus infection leads to altered production of several immunologically important cytokines, chemokines and their receptors (see below). These changes in the cytokine and chemokine production help viral replication and spread, and contribute towards pathogenesis of the disease.

Furthermore, the virus is also likely to regulated autophagic and DNA damage responses of the host cells to its advantage. Table 7 enumerates various ways by which the virus promotes its replication, evades host's responses and induces different pathologies in the host.

Effects of HHV-6 on Cytokine and Chemokine Production in Human Cells:

Cytokines and chemokines are important means of communication between cells in the body. They coordinate biological functions of different cell types. A coordinated production of cytokines is important for mounting an effective immune response against invading pathogens. Several studies show that HHV-6 causes a deregulated production of several immunologically important cytokines.

HHV-6 induces TNF-α, IL-1β, IL-15 and IL-6, from human PBMC [160,177,178]. The virus also induces IFN-α from non-T cells [179]. The virus-induced upregulation of cytokines (TNF-α, IL-1β and IL-6) was also reported in a monocytic cell line [180]. However, the virus inhibited IL-2 production from mitogen-stimulated T cells resulting in increased expression of IL-2Rα and β chains on the cell surface [181]. It also inhibited IL-12 production from LPS-stimulated human PBMC and THP-1 monocytic cells [182,183]. The inhibitory effect on the production of this cytokine was due to virus-induced IL-10 production from the PBMC. Neutralization of IL-10 increased IL-12 production in these cultures, and addition of exogenous IFN-γ increased IL-12 production [182,183]. The inhibitory effect of the virus on IL-12 production from human macrophages was due to transcriptional suppression of IL-12 p40 mRNA. No viral effect on the expression of the p35 subunit of the cytokine was observed [126]. The virus was also shown to inhibit LPS-induced IL-18 production in human PBMC [184], although in a hepatoma cell line Hep G2, the virus induced IL-18 production [185]. It is noteworthy that exogenous IL-18 reduces number of the virus-infected cells and release of extracellular virions in human PBMC cultures [184]. As stated in the previous section, the viral infection inhibits differentiation and maturation of DC. This is accompanied by decreased production of IL-12 from these cells [117]. A decreased production of IL-12 and an enhanced production of IL-10 from various cell types may result in skewing the cytokine production towards TH2-type in HHV-6 infected individuals. Indeed, in *in vivo* studies, more IL-10 and IL-4-producing than IFN-γ-producing $CD4^+$ T cells were found in the blood of HHV-6-infected individuals [186]. The virus seems to convert CD4+ T cells into IL-10 producing Regulatory T cells. This probably reflects the effects of HHV-6 via CD46, as stimulation of CD4T cells with anti-CD3 and anti-

CD46 antibodies induces them to secrete IL-10 [187]. The virus-induced cytokine changes in human cells were the result of events associated with virus entry and required no de novo protein synthesis. Furthermore, treatment of the infected cells with phosphonoacetic acid (an inhibitor of the viral DNA polymerase had no effects on the virus-induced effects on cytokine production.

A microarray study showed that HHV-6 infection of Sup-T1 cells with both A and B variants caused an increase in the expression of genes for IL-18, IL-12R, TNFR superfamily proteins, CD4, MAPK and JNK [188]. On the other hand the expression of IL-10, IL-10R and IL-14 genes was reduced in these studies. These data suggested that the virus induces proinflammatory cytokines in the infected host. It is noteworthy that microarray studies profile gene expression at a given time and do not take into account regulatory effects of the cytokines upon one another.

B variants of the virus were shown to abortively infect an astrocytoma cell line U251 and induce proinflammatory cytokines like IL-1β and TNF- α [189]. Increased concentrations of IL-6 were also found in the cerebrospinal fluid of a young child suffering from roseola and febrile seizures [190]. Such cytokines could cause tissue destruction in the CNS.

Taken together, these studies clearly demonstrate that the infection causes significant changes in cytokine production from the host cells. The virus may shift the cytokine response of the host towards TH2 type and impair induction of effective antiviral immunity.

In addition to cytokines, HHV-6 also affects chemokine production from human cells. Chemokines are small molecular weight soluble mediators like cytokines; however their main biological effect is chemotaxis. The virus has been shown to induce IL-8, RNATES and macrophage chemotactic protein (MCP)-1 from a variety of human cells including vascular endothelial cells [125,160,185]. In an *ex vivo* lymphoid tissue culture model, the virus induces CCL5/RANTES [191]. The virus also induced this chemokine from endothelial cell cultures [192]. The chemokine can bind with CCR1, CCR3 and CCR5 and downregulate the expression of these receptors on the cell surface. Furthermore, the chemokine can also prevent HIV entry into target cells. By inducing CCL5, the virus recruits new cellular targets to the site of infection.

The virus upregulated CCR-7 (Epstein Barr virus-induced protein-1; EBI-1) in $CD4^+$ human T cells [193]. This may increase chemotactic mobility of the virus-infected cells and hence spreading of the virus in the body. The virus, however, decreases expression of CXCR4 in these cells as well in established T cell lines [98,194]. The virus decreases the expression of the CXCR4 gene by increasing the association of the transcriptional repressor Yin-Yang-1 with the gene promoter. This happens due to decreased binding of the repressor with c-Myc [194]. This reduced expression of the chemokine receptor has implications for their susceptibility to infection with HIV-1 (see below).

In addition to deregulating production of cytokines and chemokines, HHV-6 also modulates activities of many key enzymes in the infected cells. For example, it induces COX-2 gene expression and production of PGE-2 from the infected monocytes and macrophages within hours of the infection. The effect was ascribed to the viral antigen IE-2 and

stimulation of the COX-2 gene promoter via increased production of cAMP and AP-1 [54]. In this study, the exogenous PGE2 enhanced replication of the virus.

It should be noted that cytokine production is tightly controlled in human cells and may vary depending upon the cell type, its state of activation and differentiation, the presence of other cytokines and growth factors in the culture media. Thus it may not be surprising that different researchers may report discordant results concerning the impact of HHV-6 on a given cytokine. Differences in experimental conditions may also contribute to discordant results. However, it is noteworthy that HHV-6 has the potential of causing dysregulated production of a wide range of cytokines. This cytokine dysregulation may adversely affect induction of an effective antiviral immune response from the host.

HHV-6 AND APOPTOSIS:

It has been demonstrated that HHV-6 may induce apoptosis in human cells. In the virus-infected cord blood mononuclear cells (CBMC) apoptotic cells were shown to be CD+4 T cells expressing viral antigens [195]. The workers showed that Fas and TNF-α were not involved in the virus-induced apoptosis. In another study involving a continuously growing $CD4^+$ human T cell line JJHAN, both A and B variants induced apoptosis, which was augmented by TNF-α and Fas-specific antibodies [196]. The virus induced increased expression of TNFR1 on the cell surface. More importantly the workers showed that the apoptotic cells were free from the viral antigens and virus-free culture supernatants from the infected cells could induce apoptosis in uninfected JJHAN cells [196]. These data suggest that indirect mechanisms (soluble FasL and TNF-α) and not the virus *per se,* were responsible for this apoptosis. The viral infection was shown to cause increased expression of p53 and p21 in CBMC [197]. Variant A caused more pronounced increase in p53 expression and apoptosis in the infected cells as compared with B variants of the virus [176]. The apoptosis may result from failure of the infected cells to exit G2. There is evidence that the virus may also be causing apoptosis of CD4+ T cells in acute infection. The CD4+ T cells from HHV-6 infected patients underwent enhanced apoptosis when cultured ex vivo [198]. More recently, it was demonstrated that for both CD4 and CD8+ T cell types. the virus (especially A variants) infects naïve and central memory T cells, and induces their apoptosis via TNF-α, but induces proliferation in effector memory T cells [160]. Thus, different subsets of T cells may be affected differentially by the virus-induced apoptosis. The virus-induced apoptosis may play a role in its pathology. It is noteworthy that HHV-6 has been associated with Kikuchi-Fujimoto's disease that is characterized by enhanced apoptosis of lymphocytes and macrophages [199].

HHV-6 AND CELL CYCLE:

The virus induces cell cycle arrest in G2 phase in CBMC [176]. The researchers showed that *de novo* synthesized IE viral proteins induced

this cell cycle arrest. The infected cells accumulated p53, Tyr 15-phosphorylated CDK1, cyclin B and cyclin A. The arrest occurred independent of p53 and its effector molecules p21 and 14-3-3. The virus, however, inhibited proliferation in cells of the nervous system (both murine and human glial precursor cells) and arrested them in G1/S phase [124,200]. The IE gene expression was sufficient to cause these effects. The infected cells seem to mount a genotoxic response evidenced by the phosphorylation of p53, ATM and CHK2. The A variant GS was shown to inhibit mitogen-induced proliferation of human PBMC and primary CD4+ T cells [181,201]. The infected cells accumulated preferentially in G1[181]. The B variants were also shown to inhibit proliferation and cause cell cycle arrest at G1/S and G2/M transitions [202,203]. The virus induced phosphorylation of p53 at serine 20 and serine 15. The phosphorylated p53 could bind transcriptional co-activator p300. The phosphorylation at serine 15 was inhibited by caffeine, an inhibitor of ATM, whereas the phosphorylation at serine 20 was inhibited by caseine kinase (CK)-1 inhibitor, D4476 [202]. It is noteworthy p53 is phosphorylated at serine 20 by CHK2 after DNA damage. The virus thus uses an alternate kinase to phosphorylate p53. Interestingly, most of the arrested cells were not undergoing apoptosis. In Sup T1 cells, both A and B variants induced elevated expression of E2F1, which accumulated in both nuclear and cytoplasmic fractions [204]. The factor remained inside nucleus in the mock-infected cells. Despite complexing less with dephosphorylated pRB, E2F1 target genes (cyclin E and MCM5) were less expressed in the virus-infected and arrested cells [204].

It is noteworthy that HHV-6 encodes two viral proteins (DR 7 and pU14), which can bind and inactivate p53. As mentioned earlier DR 7 is an E and pU14 is a late viral antigen. pU14 also recruits p53 into virions [205]. These proteins are likely to sequester p53 and abrogate the effects of the infection-induced p53 activation. Since pU14 recruits p53 into virions, the virus infected cells may express it as early as 4 hours post-infection. Due to inactivation and sequestration of p53, the virus-infected cells become resistant to UV-induced apoptosis [206]. In non-productively infected cells, the expression of the viral inactivators of p53 may be too low. Under these conditions, activated p53 may cause cell cycle arrest. The arrested cells may undergo apoptosis, continue progression with slower kinetics or allow full round of viral replication and then die due a combination of necrosis and apoptosis.

REPLICATION

As stated above, HHV-6 enters target cells by pH-dependent endocytosis. Chloroquine can effectively inhibit *in vitro* infection of different cell lines and PHA blasts [106]. Antibodies against gH, gL and gQ can inhibit the infection process. Membrane lipid rafts play an important role in viral entry. Their destruction by depleting cholesterol from cell membranes or from virions by methyl beta-cyclodextrin inhibits viral entry into target cells. The infection reorganizes the cell membrane causing a relocation of the viral receptor, CD46, to the lipid rafts [207,208]. The incoming nucleocapsid is likely transported across microtubules to the nuclear pore

complex. The process may be similar to those described for HCMV [209] and HSV-1 [210] (reviewed in [211]. In these viruses, incoming nucleocapsids recruit via some of their tegument and capsid proteins microtubule-binding motor proteins like dynein, dynactin and kinesins. The nucleocapsid movement results from a tug of war between forces pulling towards positive and negative ends of the microtubules. The virus replication occurs in the nucleus of the host cells. Because of their relatively large genomes, Herpesviruses can afford to encode various proteins that act as enzymes involved in nucleic acid metabolism, DNA synthesis, cell cycle progression, etc. During replication, viral genes are expressed temporally in an exact sequence. They are classified into Immediate Early (IE or α), early (E or β) and late (L or γ) categories. The IE genes are transcribed immediately after viral entry into target cells without *de novo* protein synthesis by using transcriptional and machinery of the infected cells. Their expression is not inhibited by inhibitors of protein synthesis like cyclohexamide or emetin. They include U42, U81, U86, U89, U90, U94, U95 and U73, etc (see Table 5 for important viral genes and their products). Of the seven genes which are present only in B variant genomes, three (B3, B6 and B7) are also expressed without requiring de novo protein synthesis [57]. All the proteins and factors needed for their expression are present in the virions or host cells. The IE gene products are a prerequisite for the expression of E genes identified as B5, U7, U8, U9, U19, U31, U41, U51, U53 and U73. Their expression is sensitive to the inhibitors of protein synthesis, but not to inhibitors of the viral DNA polymerase like phosphonoacetic acid. The E gene products are needed for the metabolism and replication of viral DNA. L genes are encoded after E genes and their expression is sensitive to the inhibitors of protein synthesis and viral DNA polymerase. Their products are needed for the assembly and release of virions from the infected cells. They encode the viral envelope glycoproteins, tegument and capsid proteins. They include U27, U29, U36, U37, U47, and U54. Some immune modulating proteins, e.g., a chemokine encoded by the U83 is also encoded late [84]. Differential splicing, multiple transcriptional start sites an post translational modifications underlie the complex temporal expression patterns of IE, E and L proteins of HHV-6 [212]. This classification of the viral genes into three classes is in fact an oversimplification. For example E genes in HCMV, which is closely related to HHV-6, are divided into β1 (Early) and β2 (Early Late) groups. Similarly Late genes are dvided into γ1 (Leaky Late) and γ2 (True Late) genes [213]. The expression of γ1 genes is reduced but not inhibited by inhibitors of DNA polymerase. Figure 4 illustrates the steps involved in the replication of HHV-6.

The viral DNA replication needs an origin-binding protein (OBP) encoded by U73. It binds to the origin of lytic replication (Ori-lyt) present almost in the middle of the viral genome. A and B variants differ in their pac and Ori-lyt sequences [4,12]. The OBP denatures a portion of the double-stranded circular viral genome and is maintained by the helicase/primase complex encoded by the U43, U74 and U77 genes. They also provide RNA primers for the lagging strand DNA synthesis. The major DNA-binding protein encoded by the U41 binds the single

stranded DNA in the replication bubble until the viral DNA polymerase catalyses the synthesis of second DNA strand. The U27 gene product encodes the processivity factor, which helps the polymerase and increases DNA synthesis [214]. The replication of viral DNA leads to the formation of a rolling circle intermediate, which consists of a long head-to-tail concatemer. Specific viral packaging and cleavage proteins cleave and encapsidate viral DNA by recognizing the packaging (pac) sequences present at the end of the genome. The viral DNA is packaged into pre-formed capsids via portal vertex: one of the 12 vertices of the capsid containing a molecular pore formed by one or more viral proteins. The viral capsids bud from the inner nuclear lamellae. During this process, they acquire an envelope without viral glycoproteins, which present on the surface of mature virions as spikes. They de-envelope as they exit from outer nuclear membrane into the cytoplasm and re-envelope upon budding into the plasma membranes studded with viral glycoproteins [121,215-217]. This long-accepted paradigm of envelopment, de-envelopment and re-envelopment of Herpesviral capsids has been challenged [218-220]. The authors observed that newly formed capsids may also exit nucleus via impaired nuclear pores. However, impaired or enlarged nuclear pores have not been reported in HHV-6-infected cells.

The sites where capsids acquire tegument is controversial in the replication of Herpesviruses. In the case of HHV-6 infection, large intranuclear membranous structures called tegusomes, which developed de novo as cytoplasmic invaginations, were observed containing tegumented capsids [215]. However, these structures were not observed in HSB-2 cell line infected with GS strain; instead the tegumented capsids were observed only in the cytoplasm [216]. All the intranuclear capsids in the nucleoplasm outside these tegusomes were found to be naked. Tegusomes also occur in the cytoplasm of the infected cells. It is noteworthy that tegument proteins can be found in the nucleus, perinuclear space, cytoplasm and at sites of secondary envelopment, it is believed that tegumentation is a continuous process that starts in the nucleus and ends at the budding of the capsids into TGN-derived vesicles. The capsids may also acquire some tegument proteins in the nucleus despite the fact that this organelle has no visible tegusome [121].

Apart from tegusomes, two morphological structures appear de novo in HHV-6-infected cells: annulate lamellae (AL) and prominent multivesicular bodies (MVB). The late viral glycoproteins preferentially accumulate in AL. The AL appear as lateral extensions from endoplasmic reticulum and Golgi and are more prominent in the cells infected with A variants. They represent sites of O-glycosylation for viral glycoproteins. The AL can be seen in HHV-6-infected HSB-2 and Sup-T1 cells within 72 hours post infection when the late glycoprotein gp116 appears [121,221]. The capsids bud into secretory vesicles that arise from trans-Golgi network (TGN) and have characteristics of both endosomes and TGN. They contain mature viruses and develop several internal vesicles like MVB [222]. The internal vesicles as well as limiting membrane of the MVB contain late viral glycoproteins. The capsids bud into lipid raft-rich areas of the membranes. The lipid-raft-specific ganglioside GM-1 and cholesterols are incorporated into viral envelopes [223,224]. The viral

proteins become sequentially glycosylated as the enveloped virions contained within the MVB are transported to the extracellular space via exocytosis. This causes the release of virions along with internal vesicles (exosomes) to the exterior of the cell [222,224]. It is noteworthy that interfering with the process of MVB biogenesis inhibits assembly and release of the Herpesviral virions [225]. The virions, like other enveloped viruses, incorporate several cellular proteins like CD46, ezrin, clathrin, Tsg101, CD63, etc into their lipid bilayer [222,226]. The virus-associated cellular proteins have implications for autoimmunity.

Viral envelope glycoproteins (e.g., gp 116 and gp 102/85) can be detected in TGN, MVB and newly formed AL but not on the surface of virus-infected cells [216,221,227]. Consequently, the infected cells are protected from immune attack by antiviral antibodies.

The virus-induced morphological changes may occur in the infected cells to variable degrees depending upon cell type and the viral variant. For example, A variants induce more prominent annulate lamellae than B variants in the infected cells. However, they induce no such structures in astrocytes [121]. Similarly, prominent tegusomes appear in the nucleus of thymocytes infected with B variants, but not in HSB-2 cells infected with A variants [121,215,216]. The infected cells enlarge and their nuclei become lobulated. These cytopathic effects occur more prominently and with earlier kinetics in cells infected with A variants [121].

VIRAL PERSISTENCE

Like other Herpesviruses, the infected host is never able to get rid off HHV-6. The virus persists in the host for rest of his/her life. The host becomes life-long carrier of the virus and sheds it continuously or intermittently in his/her saliva. The virus can persist in the host by two mechanisms: by low-level chronic replication and by undergoing latency. The virus undergoes low-level chronic replication in brain and salivary glands [228,229]. The virus produced in the salivary glands is then shed in saliva. Latency usually occurs in monocytes, vascular endothelial cells and bone marrow progenitor cells [111,120].

VIRAL LATENCY

It is noteworthy that Herpesviruses usually cause latent infections in their natural hosts. The viruses become latent in order to escape host's immune responses. The exact molecular basis of the latency is not known. During the latent infection, the viral genome exists in a circular form (episome) and expresses only a very limited number of genes. As discussed above, one of the genes expressed abundantly in latent HHV-6 infections is U94 [42]. Interestingly a rat CMV also encodes a Rep-like gene [230]. In addition to U94, four other transcripts have been identified in latent HHV-6 infections. These latency-associated transcripts (LAT) shared protein-coding sequences with IE-1 and IE-2 ORF but differ from the transcripts, which originate from these ORF during lytic infection. The LAT have different initiation sites and 5' UTR [231]. They are expressed in a small %age of latently infected cells. Their expression

increases both *in vivo* and *in vitro* before viral reactivation [232]. HHV-6 can also exist as a latent infection by integrating its genome in human chromosomes (see the section on viral integration). The latent Herpesvirus infections become reactivated in humans under stress, immunodeficiency, etc. In this connection, two cell lines, SAS413 and SAS527, have been developed from a patient undergoing blast crisis of CML [233]. B variants of HHV-6 can infect these cells latently. The infection can be reactivated with PMA in these cells with production of cytopathic effects. The cells provide a useful in vitro model for studying how latent HHV-6 is reactivated from latency. It is commonly believed that immunosuppression usually leads to viral reactivation. However, they also occur in immunocompetent but critically ill or even in apparently healthy persons. Ward et al have described high level persistent A and B reactivations (viral DNA in plasma) in healthy immunocompetent persons [234]. The authors ascribed these high level viral replications to congenital infections.

Upon reactivation, the production of virions through enhancement of lytic cycle increases. Upon reactivation, many tissues in the body, e.g., salivary glands, brain, liver, tonsils, endothelial cells, lungs, skin, etc are infected in humans and give rise to different clinical manifestations. The manifestations may involve one or more organs of the body (see below).

VIRAL INTEGRATION

A number of studies have demonstrated that HHV-6 can integrate in the host genome both *in vivo* and *in vitro*. The integration occurs within or near telomeric sequences of the host chromosomes. Integration of the viral genome at the ends of human chromosomes 1, 17 and 22 has been documented [235-238]. It has been suggested that the presence of telomere like repeats at the termini of the viral genome may play a role in the site-specific integration. Usually one copy of the genome is integrated in one cell and the virus is latent as no IE transcripts could be detected [239]. The integration of viral DNA in host genome has implications for congenital transmission to newborn children and bone marrow transplantation. If integrated in germ cells, the virus could be transmitted congenitally to newborn children. It has been estimated that 1-2% children may be infected via this route. The conclusion was drawn as 57 out of 5638 cord blood samples were found positive for the viral DNA. All children remained healthy despite signs of active viral replication [240]. The incidence of chromosomal integration of the HHV-6 DNA in Japanese population was estimated to be about 0.2% [241]. Similarly, the infection could be transmitted to bone marrow recipients if the viral genome has been integrated in the hematopoietic progenitor cells of the donor.

Because of its genomic integration and latency in several cells and tissues of the body, HHV-6 can be easily transmitted in hospital settings via blood transfusions, and transplantation of solid organs and stem cells [242]. Clark et al identified a donor who had HHV-6 integrated into chromosome 17p13.3. The integrated viral genome was also found in the recipient after, but not before, the transplant. The viral load increased in the recipient after transplantation in parallel with the engraftment. Thus,

increased viral load in some recipients may not represent reactivation [243]. More viral DNA is found in CSF if HHV-6 is integrated in genome.

As mentioned above, U94 is a homologue of the rep genes of AAV-2. The gene products play a role in site-specific integration of the virus (reviewed in [244]). No such role has been assigned to U94 protein in the integration of HHV-6 in human genome.

DIAGNOSTIC TESTS

Several diagnostic assays have been developed and used for HHV-6. Despite these tests, clinically relevant diagnosis of the virus remains a challenge. The diagnostic tests are based on serology, cell culture, PCR, in situ hybridization, and immunohistochemistry. Each test has its merits and limitations (see Table 8). The serological assays include indirect immunofluorescence assay (IFA) and ELISA [245]. In the IFA, HHV-6-specific antibodies can be detected by using HHV-6-infected cells in test sera. In ELISA, lysates from virions or HHV-6-infected cells are used to detect antiviral antibody titres in test sera. The test is quantitative. These assays can be modified to detect antiviral antibodies of IgM and IgG isotypes. The acute primary infections in children can be diagnosed either by positivity for IgM or by more than four fold increase in IgG titres in sera collected at 2-3 weeks time intervals. False positive and false negative serological test results have been reported. Therefore it is important to substantiate these results by other assays, e.g., by cell culture method. A limitation of the serological assays is that they cannot distinguish between latent infection and active viral infections. Furthermore, antibodies cross reacting with HHV-7 and HCMV remain a confounding factor. Unfortunately, in immunocompromised patients with chronic active replication, serological assays do not provide much information.

HHV-6 can also be diagnosed in clinical samples by their co-culture with IL-2 and PHA-activated lymphoblasts obtained from peripheral or cord blood of healthy donors [246]. The presence of the virus in the co-cultures could be identified by characteristic cytopathic effects of the infection, which can be confirmed by HHV-6-specific monoclonal antibodies by IFA or Wesrtern blots [94]. A positive test, however, cannot distinguish between primary, reactivated latent or active lytic infection. It may simply also indicate the presence of secreted virus in the clinical samples [32].

There has been the tendency of detecting HHV-6 in clinical samples by PCR. Real time PCR, with appropriate controls, gives quantitative results, which could distinguish between latent and active viral infections. In a relatively recent study, Flamand et al [247] compared the ability of different PCR-based methods for their ability to detect spiked viral DNA in serum samples. They found that three Taqman-based Real Time PCR methods were able to quantify viral DNA very close to the spiked amounts. The detection of increased viral load in the PBL or viral DNA in peripheral blood in the absence of viral DNA in the saliva indicates a primary infection [248]. It was surmised from a cohort of 143 children hospitalized for undifferentiated febrile illness that the detection

of viral DNA in whole blood in the absence of virus-specific antibodies in the plasma was the most reliable criteria for diagnosing a primary infection. The detection of viral DNA in the whole blood and the viral load of more than 3.3 $\log^{10}$ copies per 5 ul predicted primary infections with 90% and 100% sensitivity, respectively [249]. It was also found that the presence of maternal antibodies could confound serology-based diagnosis until the age of three months.

More recently, the diagnosis of active HHV-6 infection has been based upon the viral load present in the PBMC, similar to the one used for the diagnosis of HCMV. The HHV-6 viremia has been shown to correlate with serological response of the host [250]. The antibodies directed against the structural (late) viral antigens have been used to detect localized infections in various tissues by immunohistochemistry [251]. Viral DNA could also be detected in tissues by in situ hybridization [252]. Again the detection of viral antigens or of DNA in tissues does not necessarily mean that the virus is actively involved in the disease process. However, these methods may help in differentiation between latent and active viral infections.

The distinction between active latent and active HHV-6 infections has been made by RT-PCR targeting spliced mRNA for a number of genes. For example, this method has been used to detect mRNA for IE (U16/17, U89/90) and late (U60/66) genes of the virus for active HHV-6 infection [253].

Because of significant differences between biological characteristics of A and B variants of the virus, it is highly desirable to make a variant-specific diagnosis. Fortunately A and B variants have characteristic restriction endonuclease profiles. This has allowed the development and use of Restriction-Fragment-Length Polymorphism (RFLP) assays for diagnosing clinical isolates of the virus [10,254]. Furthermore, several variant-specific monoclonal antibodies have been developed that could be used in IFA for diagnosis of A, B or mixed infections.

CLINICAL MANIFESTATIONS

The virus acts as an opportunistic pathogen. Primary infections are often mild and remain latent in healthy individuals. Chronic or intermittent reactivations of the infection in immunocompromised individuals may cause a variety of disease conditions and clinical symptoms. The virus-induced disease conditions are discussed below:

A. ROSEOLA INFANTUM

It is also named as Exanthem subitum or the sixth disease. The clinical syndrome was described for the first time by Zahorsky [255]. However, it was in 1988 that a group of Japanese researchers established its etiology with HHV-6 [246]. Only a fraction of the infected children develop roseola, which is characterized by the presence of a high fever and the development of a cutaneous eruption [256]. The disease occurs exclusively in children of less than three years of age. The fever usually lasts for 3-5 days. It is followed by the appearance of cutaneous eruptions. These

maculopapular eruptions appear 48 hours after the defervescence and are frequently mistaken for allergic reactions to medication [257]. As mentioned earlier almost all cases of roseola arise from infection with B variants of HHV-6 [258]. It is noteworthy that the febrile illnesses in children caused by HHV-6 are frequent reasons for visits to hospital emergencies. It has been estimated that up to one third of the young children, who suffer from febrile illness of unknown cause or from fever caused by otitis and are brought to the emergency room, are due to primary infections with HHV-6 [6,259,260]. A study followed 227 children from birth to two years of age. Of these children 94% became infected with the virus of which 58% showed fever, 24% roseola, fuzziness, rhinorrhoea, cough, vomiting and rashes. About 13% of the primary HHV-6 infections in children younger than 2 years of age result in convulsions and seizures [260]. The viral infections with HHV-6 may account for 20-30% of the febrile seizures occurring in children. It is believed that viral invasion of the CNS and its later reactivations are implicated in the pathogenesis of recurrent febrile seizures in children [261]. More recently, about 8% of the 156 children hospitalized for severe febrile illness or encephalitis in Great Britain had primary HHV-6 infection [262]. Interestingly, in a small percentage of all individuals, both A and B variant DNA persisted and were detected in the serum [234]. Suga et al have suggested that the febrile seizures associated with primary HHV-6 infections could be more severe, atypical, partial and prolonged or recurrent. They could be fatal and cause paralysis. They may enhance risk of developing epilepsy [263]. However, in another study on HHV-6-caused febrile seizures in children, it was shown that the infection does not pose more risk of recurrent seizures in children as compared with seizures due to other causes [264]. A later study suggested that the febrile seizures associated with primary HHV-6 infection correlated with reduced risk of developing recurrent seizures [265]. Differences in results from these studies could be due to differences in diagnosing the infection and/or due to biased sampling of the study participants.

B. MENINGITIS AND ENCEPHALITIS

Although HHV-6, especially A variants, show neurotropism, the ubiquitous nature of the virus makes it difficult to assess its role in the pathophysiology of brain. Up to 32% of the brain tissues obtained from the autopsies of healthy persons could be positive for HHV-6 DNA when detected by PCR. A variants occur three times more frequently than B variants [251]. Nevertheless, several studies have implicated HHV-6 as an etiological agent in meningitis and encephalitis in immunocompromised persons as well as in healthy children and adults [266,267]. In these studies the diagnosis of encephalitis was established by the detection of viral DNA sequences in the cerebrospinal fluid (CSF) or in the brain by PCR in the absence of other etiologies. The virus may directly invade brain and cause encephalitis. It is noteworthy that the virus has been shown to infect human astrocytes, oligodendrocytes and microglia in *in vitro* studies [122,268]. The infection may become latent in the CNS, reactivate later in hippocampus and cause CNS disease (reviewed in [269]). However

HHV-6-associated encephalitis and encephalopathies have been reported without any inflammatory changes and without the detection of viral DNA sequences in the brain [270]. The fatal cases of encephalitis caused by coinfections with HHV-6 and HSV-1 have also been documented [271]. In a cohort of 138 patients with focal encephalitis of unknown etiology and who were also negative for other Herpesvirus DNA and antibodies, 9 (6.5%) were detected positive by PCR for the presence of HHV-6 DNA in their CSF. Surprisingly DNA sequencing showed that the viruses in the brain tissues were of B variants[266]. Another study showed that among 50 patients with encephalitis or meningitis, 20% show HHV-6 specific antibodies or its antigens in CSF [272].

The prognosis of HHV-6-induced encephalitis is generally good. The sequelae like hemiparesis and neurological deficits are rare. However some fatal cases have been reported [266,267]. It is yet not clear whether HHV6 is the cause of seizures. A recent study on patients with temporal lobe epilepsy showed high levels of HHV-6 DNA and viral gene expression in the lobe sections [273].

C. HHV-6 INFECTIONS IN TRANSPLANT RECIPIENTS AND IN IMMUNOSUPPRESSED PERSONS

HHV-6, like other Herpesviruses, causes latent infections in their natural hosts. The latent infections usually become reactivated in immunocompromised persons and cause different clinical manifestations, which may be organ specific or generalized. Most of these manifestations occur in transplanted patients undergoing immunosuppressive therapies. The clinical mainfestations of reactivated HHV-6 in transplantees include fever, skin eruptions, pneumonia, encephalitis, gastroenteritis and hepatitis. The incidence of these manifestations depends upon the transplanted organ, i.e., whether it is bone marrow, cord blood, liver, heart or kidney. The use of immunosuppressive regimens also affects reactivation and its consequences. The consequences of the viral reactivations may vary and may result in graft rejection, graft versus host disease (GvHD) and failure of the immune reconstitution.

The median incidence of reactivations is 48% in bone marrow transplantees and 62% in solid organ transplantees [4,7]. A vast majority of the viral infections in the transplantees is due to reactivation of latent infections with HHV-6 B variants. They usually occur 2-4 weeks post-tranplantation [274]. Primary infections could also occur in the transplanted patients due to the transplantation of an organ from an infected donor [242]. It is noteworthy that OKT3 and anti-lymphocyte globulins are frequently used in solid organ transplantees to prevent graft rejection. Their use is often associated with enhanced incidence of HHV-6 reactivation in the transplant recipients [275]. Most probably this reactivation occurs due to immunosuppression induced by these antibodies, as they may deplete antiviral T cells in the transplantees.

Reactivated HHV-6 in bone marrow transplantees may cause fever, skin eruptions, pneumonia, encephalitis, bone marrow suppression, and GvHD [32]. The fever is usually >41^{o}C and accompanied by leukopenia and skin eruptions [275-278]. Pneumonitis also occurs frequently in the transplantees

with HHV-6 reactivations [276,279]. The virus could be isolated from the broncho-alveolar lavage (BAL) fluid, lymphocytes present in the sputum, and PBMC [280,281]. The clinical significance of the detection of HHV-6 DNA in the bone marrow is not clear, as it could also be present in healthy individuals. In order to diagnose HHV-6-induced pneumonia in bone marrow transplant (BMT) recipients, qPCR has been used to determine viral load. The presence of more than 20,000 copies of the viral genome per 10 copies of the cell genome is suggestive of the reactivation [9]. Furthermore, increased titres of the anti-HHV-6 antibodies are also significantly associated with idiopathic pneumonia in BMT recipients [281]. The HHV-6-related idiopathic pneumonia seems to have better prognosis as compared to the pneumonia caused by other etiological agents in BMT recipients [280,281]. In addition to HHV-6 isolation, one must exclude that the pneumonia is not caused by other infections, e.g., HCMV. Encephalitis and encephalopathy are the most frequent clinical complications associated with HHV-6 reactivation in transplanted patients [276,282]. Isolation of the virus and/or detection of its DNA in the CSF by PCR is used to diagnose HHV-6-induced encephalitis in transplanted patients [267,283]. The virus can also be detected in the brain biopsies by immunohistochemistry [282]. It is noteworthy that both A and B variants can be detected in the affected brains. The HHV-6-induced encephalitis is characterized by headaches, seizures and changes in the mental status of the patients, abnormal movements and fever. The CSF from the affected patients may contain a higher number of mononuclear cells and inflammatory proteins. Usually MRI results are normal; however, weak cerebral lesions have been reported in a subset of patients. Antiviral therapy in these patients could reduce deaths [267]. HHV-6 has been implicated in the suppression of bone marrow and stem cells in the transplanted patients and delayed engraftment [284-286]. HHV-6 could be isolated from the blood and bone marrow biopsies. In vitro the virus suppressed bone marrow in colony forming unit assays [287]. This association between HHV-6 and bone marrow suppression is also supported by *in vitro* data documenting the suppressive effects of the virus on the proliferation of stromal cells, granulocytes, macrophages, megakaryocytic and erythroid cell lines [288,289]. The virus can infect lineage-committed $CD34^+$ hematopoietic stem cells *in vitro* and suppresses their growth and colony forming potential [290]. The virus infects these cells latently and is transmitted longitudinally to differentiated cells of multiple lineages. The infection suppresses development and maturation of these cells. The suppressive effects of the virus on bone marrow could result from direct infection of the cells as well as from indirect effects via cytokines The suppressive effects could affect all cell types, however, they are particularly pronounced against granulocytes, macrophages, megakaryocytes and erythroid lineages [291]. *In vitro,* B variants have more suppressive effects on myeloid precursors than A variants of the virus. Nevertheless, it was observed that A variants could also exert serious myelosuppressive effects after transplantation [292,293]. HHV-6 has been implicated in late engraftment of granulocytes [292]. The myelosuppressive effects of primary infections could be prevented by antiviral treatments (see below) [242].

Some of the suppressive effects observed in BMT recipients have also been observed in solid organ transplantees [294]. The transplantees may show gastroenteritis without sever GvHD. In this connection, a positive correlation was found between HHV-6 in skin biopsies and severity of the GvHD [295]. The viral reactivation may also cause rejection of the graft in solid organ transplants (SOT). HHV-6 reactivation and antigenemia have also been repoted to occur in SOT. As high as 91% of the lung and heart transplantees may show HHV-6 reactivations, whereas reactivation of other herpesviruses (HCMV and HHV-7) is relatively less frequent (50-55%). HHV-6 reactivations in SOT may also result in encephalitis and pneumonitis [296]. These reactivations usually occur within two weeks after the transplant and predominantly (upto 98%) involve B variants [297-302]. In SOT reactivations are associated frequently with encephalitis, other Herpesvirus infections, organ rejection, and mortality [299,303]. In liver transplanted patients, HHV-6 reactivations are associated with more sever form of HCV recurrences [299]. HHV-6 reactivation also poses a risk for HCMV disease in liver transplantees [304] (reviewed in [305]). Both HHV-6 and HHV-7 are involved in skin eruptions in organ transplant recipients [306].

It is noteworthy that HHV-6 infections do not occur in transplant patients in isolation. These infections are often accompanied by infections with other Herpesviruses including HHV-7, HCMV and EBV. The infections occur with a median period of 20 days for HHV-6, 26 days for HHV-7 and 36 days for HCMV [298,307]. Thus, it appears that HHV-6 infection may often cause reactivation of HHV-7 and HCMV in these patients. Furthermore, HHV-6 along with HCMV has also been implicated in the functional deterioration of the graft as well as in its rejection [307]. Co-infections with these two viruses often result in serious clinical symptoms in transplanted patients [308]. It has been observed that HHV-6-specific antibody responses and quantities of PCR-determined viral DNA correlate with HCMV-induced disease [309]. HHV-6 has also been associated with symptomatic infections with HCMV and increased risk of graft rejection. These observations could be explained by cross reactivity of antibodies for different antigens from the two viruses as well as by potential interactions between the two viruses. The HHV-6-induced immune suppression could trigger reactivation of HCMV and/or alter the natural course of HCMV infection. Its reactivation could also result in the rejection of kidney grafts.

As mentioned above, HHV-6 can infect vascular endothelial cells and induce secretion of several proinflammatory cytokines [125]. More importantly the infection prevents angiogenesis. It is suspected of causing damage to vascular endothelial cells in BMT recipients. This may also lead to the formation of thrombi.

Reactivations of HHV-6 in stem cell transplantees may also result in delayed engraftment of neutrophils. A retrospective study involving 78 stem cell recipients found correlation between virus load in the PBMC and delayed engraftment of neutrophils as well as between serious GvHD and classical manifestations of HHV-6 infection, i.e., fever, skin eruptions, etc. [300]. The researchers reported higher HHV6 load (median 1357 genome equivalent copies per one million PBMC) in these patients.

The load was undetectable in 31 immunocompetent individuals. These data suggest HHV-6 reactivation in the transplanted patients. High load in delayed neutrophil engraftment (p=0.002) or caused severe GvHD (p=0.009). High viral load (more than 1000 copies) was also associated with at least one HHV-6 related event (fever, cutaneous rash, pneumonitis, partial myelosuppression). Interestingly, no differences in the HHV-7 loads were observed in the two groups ([300]. The study suggested that HHV-7 may act as a co-factor for reactivation of HHV-6. Another study comprising 25 recipients of BMT reported a high incidence of HHV6-A DNA in plasma (24 positive in 25) than in PBMC (1 positive in 25) whereas the incidence of detecting HHV6-B in the two compartments was comparable (22 and 21 patients being positive for the viral DNA in PBL and plasma out of 25 patients, respectively). Overall 80% of the patients had two variants circulating in their plasma [310]. The frequent detection of HHV-6A in plasma but not in PBMC indicates that the virus becomes reactivated in tissues other than PBMC.

It is not clear what causes reactivation of the virus. Some studies suggest the involvement of alloreactive immune responses. This is supported by a significant association of the reactivations with grafts from unrelated donors (URD). For example, in a study involving 228 SCT recipients comprising 197 allografts and 31 autografts, 42.1% developed HHV-6 reactivations. A majority (58%) of the recipients developed interstistial or alveolar pneumonia, gastroduodenal and colorectal disease, liver disease and bone marrow suppression. The reactivation was significantly associated with GvHD (OD=5.31), EBV co-infection (OD=8.89) and with URD (OD=5.67) [311].

Cord blood transplantations (CBT) have advantages over unrelated BMT in children in causing less GvHD but having comparable rate of disease-free survival. However, they may also pose risk of HHV-6 infections. As mentioned above, about 1-2 % individuals may have the viral DNA integrated into their genomes, and hence in their cord blood cells. In one study, 362 cord blood samples were tested for EBV, HCMV, HHV-6 and HHV-8 DNA by PCR. Two of the samples tested positive for HHV-6 DNA, probably due to this integration. No other viral DNA could be detected in these samples. However, the samples were from a non-endemic area for HHV-8, therefore these results may not reflect true risk for HHV-8 for endemic areas. These data suggest that CB should be screened for HHV-6 before transplantation [312,313]. Interestingly Yamane et al [313], found that CBT, HLA mismatch between donor and recipient and low anti-HHV-6 antibody titers before transplantation were the risk factors associated with the development of DNAemia in a cohort of patients comprising 46 recipients of allogeneic marrow or peripheral blood SCT from related eleven or unrelated twenty-two donors and CBT from 13 URD. The DNAemia was observed more often in the patients with CBT (92.3% vs 30.3%; P<0.01). Three patients developed CNS disorders with detectable HHV-6 DNA in CSF. Thus monitoring HHV-6 DNA could be useful in detecting CNS disorders. HHV-6 B, but not A, reactivations occurred frequently in allogeneic bone marrow transplantees and were associated with skin rash, HCMV reactivation and hemorrhagic cystitis [314]. Early manifestations of the HHV-6B

reactivation (within hundred days after the transplant) include skin rash, hemorrhagic cystitis and HCMV antigenemia [314]. In 72 recipients, monitored for HHV-6 DNAemia by real time quantitative PCR, HHV6-A reactivation occurred only in 1 (1.4%), whereas HHV-6B was detected at least once in 47.2% (34/72) persons in 7-84 days (median of 21 days). HHV-6B reactivation had no effect on platelet and neutrophil engraftment and on mortality in this period of time. In another study involving 72 allogeneic hematopoietic stem cell transplant (HSCT) recipients, peripheral blood samples were taken before and every week after the transplantation and tested for DNAemia by nested PCR. HHV-6 DNAemia was detected in 62.5% of the recipients in 7-63 days (median 14 days) in the recipients.

Not much information is available concerning the impact of antiviral treatment on clinical symptoms associated with HHV-6 reactivation in the transplantees. One of the two encephalitis patients, who received unrelated CBT and had viral DNA in the CSF, responded to ganciclovir but again suffered from encephalitis and died [315]. Unfortunately no case controlled studies have been carried out to determine the effects anti-HHV-6 therapy in transplanted patients. Further studies are needed to evaluate whether preventive antiviral therapy against HHV-6 may be necessary to reduce viral load and their adverse consequences in transplanted recipients.

In HSCT reactivations are associated with encephalitis, bone marrow suppression and GvHD [298,300,316-321]. The reactivation-induced encephalitis is accompanied by loss of memory, depressed consciousness, confusion, encephilitus and seizures in one third of the patients [321,316].

In recent studies it was shown that in 197 allogeneic and 31 autologous transplant recipients in Germany [311], 43% patients had reactivations mostly in URD, and were associated with GvHD. In another study in the USA [302] with 110 allogeneic transplantees, 47% reactivated HHV-6 evidenced by the presence of viral DNA in the plasma. Multivariate analysis showed that lower age, steroid use and underlying disease were predictors of the reactivation. The reactivations were independently associated with delayed engraftment of platelets, GVHD, more frequent platelet transfusion needs, and overall mortality. These observations have been verified both in children and adults in other studies involving smaller number of patients ([284,322,323]). Rashes, fever, delayed engraftment were also often seen in children. These studies show that the incidence of viremia appears to be much higher in the recipients of the allogeneic bone marrow as compared to recipients of autologous ones. Furthermore, the presence of leukemia or lymphoma and low titers of anti-HHV-6 antibodies are predisposing factors to the development of high viremia after transplantation.

D. AIDS

HHV-6 reactivations also occur in HIV-infected persons. Disseminated infection with the herpesvirus was detected in a series of nine unselected AIDS patients at necropsy [324]. The two viruses preferentially target and

can co-infect the same human cell type, i.e., CD4+ T cells [61,325]. By infecting and killing CD4+ T cells, it is argued that HHV-6 promotes depletion of these cells in HIV and HHV-6 co-infected persons. It is not surprising that since its discovery in 1986, HHV-6 has been considered as a co-factor in the progression of HIV infection towards AIDS. There are many ways in which HHV-6 could enhance HIV replication and promote AIDS progression. They are described as below:

HHV-6 increases HIV replication: Two DNA fragments from HHV-6 have been reported to increase Tat-induced transcription of the HIV LTR, which depended upon the presence of certain elements in the TAR and enhancer region of HIV. The fragments (pZVH-14 and pZVB-70) were previously shown to increase tumorigenicity of HPV transforming genes [326]**.** A pZVB-70 subfragment encoded a putative IE protein that enhanced HIV LTR via NF-κB site [63]. It was also shown that *in vitro*, the HHV-6 gene DR7-encoded protein can also activate HIV LTR [31]. Several other HHV-6 encoded transactivator proteins have been demonstrated to activate HIV LTR in *in vitro* studies. They include IE-B proteins (encoded by the U16/17 region [61,64,327], PU3 (a positional homologue of the HCMV UL24; [29,31], IE-1 [51,52]. Tat interacts synergistically with these HHV6 proteins and increases HIV replication [326,328]**.** HIV was shown to enter and infect human syntiotrophoblass. A co-infection of these cells with HHV-6 induced full replication of HIV without affecting replication of the herpesvirus. The stimulation of HIV depended upon IE gene of HHV-6 [329]. More recently, HHV-6 was shown to increase HIV replication in a chronically infected human T cell line [330]**.** The enhancement seemed to be caused by IE genes of HH6. It is noteworthy that both viruses can productively co-infect the same cell type and accelerate cell death [61]. Given that HIV-infected persons are also infected with HHV-6, the latter virus was considered to enhance HIV replication and enhance progression of HIV infection towards AIDS.

HHV-6 increases the pool of HIV-infectible cells: It has been shown that HHV-6 activates CD4 promoter and induces expression of this molecule on the surface of otherwise CD4-negative cells. The products of two ORF U86 and U89 present in the IE-A region and possibly other unknown ORF products of HHV-6 were implicated in this activation [52]. Indeed it was shown that the virus infects NK cells, CD8+ T cells and γδ-T cells, renders them CD4-positive and hence susceptible to infection with HIV [108,112]. Even hematopoietic stem cell lines become susceptible to infection with HIV after infection with HHV-6 [331]. It was argued that by increasing the pool of HIV-infectable cells in the body, HHV-6 may be accelerating the progression of HIV infection towards AIDS.

By inducing immunosuppression: HHV-6 suppresses immune responses of the host in many ways. It adversely affects functional activities of CD4+ T cells, dendritic cells, macrophages, etc directly or indirectly via deregulated expression of cytokines and chemokines (detailed in the section on pathogenesis). The virus-induced immunosuppression may compromise ability of the host to mount an effective immune response against HIV-1.

By inducing cytokines that increase HIV replication: HHV-6 is known to modulate cytokine production in human cells. The virus induces

production of TNF-α, suppresses production of IFN-γ from human cells [178,182]. The former cytokine enhances HIV replication by upregulating NF-κB, and the latter one induces antiviral state in human cells [332]. The virus-induced cytokine milieu is more conducive to HIV replication.

In the face of these potential mechanisms by which HHV-6 can enhance HIV replication, several studies have demonstrated that the virus could also inhibit HIV replication in a variety of different cell types. The herpesvirus was shown to inhibit HIV replication in human PBMC and CD4+ T cells, especially when the cells were infected with HHV-6 at higher MOI as compared with HIV [333]. It was also described to suppress HIV replication in co-infected DC [334]. The potential mechanisms by which HHV-6 can inhibit HIV replication include:

By encoding HIV suppressive proteins: HHV-6 encodes several proteins, which suppress LTR-induced HIV replication. For example the product of the U94 gene, which is implicated in maintaining HHV-6 in latent state, strongly inhibits HIV replication in human cells [4,45].

By inducing chemokines and virokines that inhibit HIV entry: HHV-6 not only induces C-C chemokines (e.g., RANTES) from host cells, it also encodes a virokine (U83), which acts as a C-C chemokine and bind CCR5 and CCR2 with high affinity [110,191,192]. These two chemokine receptors act as co-receptors and are used by macrophage-tropic (M-tropic) HIV strains to gain entry into human cells. Indeed, U83 from HHV-6A variants effectively inhibits entry of M-tropic HIV-1 into CCR5-positive human cells [86]. It is noteworthy that a majority of new HIV infections is initiated by M-tropic HIV. A splice variant of U83 is expressed early in HHV-6 infection and also blocks HIV entry in *in vitro* assays.

By decreasing the expression of CXCR4 on human cells: CXCR4 is used as an essential co-receptor for entry of T cell-tropic (T-tropic) HIV strains into human cells. Some studies suggest that HHV-6 infection downregulates the expression of this receptor on human cells [98,194]. In another study researchers propagated HIV in an ex-vivo model of human lymphoid tissue. They found that co-infection with HHV-6 did not affect the expression of CXCR4 and CCR5. Nevertheless they found an inhibitory effect of HHV-6 on T-tropic but not on M-tropic HIV replication [191].

Similar to the results obtained from *in vitro* studies, co-infections of the two viruses in animal models also gave controversial results. In the SCID-Hu Thy/Liver mouse model, co-infection with HHV-6 and HIV-1 had no effect on each other's replication and cytopathic effects [335]. It is noteworthy that in this mouse model, human thymus and liver tissues are implanted in SCID mice under kidney capsule. The human fetal tissues fuse and develop as a unique Thy/Liv organ, which is equivalent to human thymus. The organ supports long-term human lymphopoiesis. Such SCID-hu mice can be infected with HIV-1 as well as with HHV-6 by direct intrathymic viral injections. Both viruses replicate in thymus in these mice. HHV-6 alone infects intrathymic T-progenitor cells and depletes Thy/Liv of thymocytes [139]. In contrast to these studies in mice, a co-infection of chimpanzee cells with the two viruses enhanced

cytopathic effects and accelerated cell death [131]. However it is noteworthy that HIV does not cause AIDS-like disease in chimpanzees. The virus also increased AIDS progression in SIV-infected pig-tailed macaques. The co-infected animals develop higher titers of the virus and exhibit more rapid loss of CD4 and CD8+ T cells [133]. It is noteworthy that SIV causes acute infections in these animals whereas HIV-1 rather causes chronic infections in humans.

E. STUDIES IN HIV AND HHV-6 CO-INFECTED INDIVIDUALS:

The effects of HHV-6 infection on the course of HIV infection in HIV-infected humans are not very clear and present a mixed picture. Clinical data from HIV-infected adult patients showed that HHV-6 co-infection does not result in increased progression towards AIDS [336]. In this study involving 32 HIV and HHV-6 co-infected persons, the workers found higher viral loads and higher frequency of HHV-6 infection in the patients with higher CD4+ T cell counts. HHV-6 antibody titers and viral loads in saliva did not correlate with CD4+ T cells counts or with stage of the disease.

In another study 227 children, born to HIV-seropositive mothers, were monitored for HIV and HHV-6 infections, and CD4+ T cell counts [337]. Interestingly HIV-uninfected children had higher cumulative rates of infection at 6 and 12 months of age as compared to HIV-infected children. In the children, who were infected vertically with HIV, concomitant infection with HHV-6 caused more rapid progression towards AIDS. The study however included only 10 children coinfected with HIV and HHV-6, and 12 children infected with HIV-1 only, and were followed for one year [337]. The study suggests that co-infection with HHV-6 increases the level of HIV replication in children.

Studies conducted to determine effects of HIV on HHV-6 replication have also given controversial results. In one study, HIV infection as well as its protein Tat was reported to increase HHV-6 production in human cord blood lymphocytes and in a continuous CD4+ T cell line J-JHAN [338]. However, Tat was reported to inhibit HHV-6 production in another T cell line Jurkat. This was observed despite a synergistic activation of HIV LTR by Tat and HHV-6 infection [328]. In an in vivo study, HHV-6 titers were not different between HHV-6 infected HIV seropositives and HHV-6 non-infected seropositives. The titers of anti-HHV-6 antibodies tended to decrease with time in HIV-infected persons. Furthermore the infections with HHV-6 were not associated with progression of HIV infection to AIDS [339].

In one study, HHV-6 viral load was determined by PCR in 151 autopsy specimens coming from 11 HIV-infected AIDS patients. The load correlated with the HIV viremia suggesting mutual enhancement of viruses [340]. In a similar study HHV-6 antigen was detected in all lymph nodes of HIV–infected persons, and active HHV-6 infection was found in all AIDS patients [341].

HHV-6 shedding tended to be less frequent in HIV-infected than in HIV-seronegative normal subjects [342]. The study included 44 HIV-infected and 15 HIV-seronegative healthy controls. Another study [343] employed

359 HIV-infected subjects found that HHV-8 and HSV-2, but not HHV-6, were associated with progression towards AIDS. Similar finding were reported from a cohort of 120 HIV-infected persons[344]. These researchers found that HHV-6 (and HHV–7) infections were quite infrequent in HIV-infected persons at any stage of infection. The herpesviral DNA in plasma frequently reacted with EBV, HCMV or HHV-8 but not with HHV-6 or HHV-7. These data suggest indeed that HHV-6 is an opportunistic pathogen.

Active HHV-6 in HIV seropositives has been associated with encephalitis [345,346] and pneumonitis [347] but not consistently [348,349]. In most HIV-seropositives, HHV-6 causes no major complication or any specific clinical syndrome and does not alter course of the infection [339]. It is noteworthy that HCMV infections have been frequently associated with retinitis in HIV-infected AIDS patients (reviewed in [350]). In most cases of AIDS-associated retinitis, retinae are also infected with HHV-6. In some cases HHV-6 transcripts could be detected in the absence of HCMV or any other Herpesvirus. These data suggest that HHV-6 not only may predispose HIV-infected patients to HCMV infection, but may also be etiologically linked to retinitis in these patients [351,352].

In conclusion, HHV-6 infection may modulate HIV-infection in the co-infected persons. Whether the herpesvirus enhances or inhibits HIV replication and disease progression may depend on several factors. The factors include the degree of immunosuppression, predominant state of HHV-6 infection (latent or lytic), relative burdens of the two infections, and the stage of HIV infection. A predominantly latent HHV-6 infection may inhibit and predominantly lytic one may enhance HIV-1 replication. Late in the course of HIV infection, when CD4+ T cells become severely depleted, HHV-6 replication and viral loads may decrease simply due to less availability of fully permissible target cells. Ironically, an opportunistic pathogen like HHV-6, which was considered to be a major factor in causing progression of HIV-infections towards AIDS, turned out to possess genes whose products strongly inhibit entry and replication of HIV. These gene products may prove important novel anti-HIV therapeutics.

F. CENTRAL NERVOUS SYSTEM DISEASES

Brain is an important organ for both active and latent infections of HHV-6. Primary infections with HHV-6 are characterized by high fever, which is often accompanied by seizures. The viral reactivations in immunocompromised patients are often manifested as encephalitis and/or encephalopathy. Active HHV-6 infections may cause serious CNS disease both in immunocompromised and immunocompetent adults. The disease is charecterized by fulminant multifocal demyelinations [282,353]. The virus invades brain and it is testified by the fact that viral DNA can be frequently detected in the specimens taken from diverse regions of the brain. About 32 to 85% of the brains are detected positive for vial DNA by PCR [273,354,355]. Both A and B variants have been detected in brains with frequencies that reflect their seroprevalence; B being three fold more frequent than A variants. The two variants may infect different

regions of the brain, at least in some patients [356]. In dually infected patients, only A variants are present in the CSF. This suggests that these variants are more neurotropic and preferentially replicate in brain tissues. In addition to its association with multiple sclerosis, HHV-6 has also been suggested to act as a co-factor in the development of progressive multifocal leukoencephalopathy (PML), which is a demyelinating disease of the CNS occurring in individuals with deteriorated cell-mediated immunity. It is usually caused by Poliomavirus JC. However, HHV-6 cooperates with JCV to trigger PML ([357]; reviewed in [6]). More recently, the reactivation of these viruses and the development of PML have been observed after using immunosuppressive drugs in patients suffering from Crohn's disease or multiple sclerosis [358,359].

G. OTHER CLINICAL ASSOCIATIONS

HHV-6 has been associated with a number of diseases. They are discussed below:

MULTIPLE SCLEROSIS (MS)

MS is the most common demyelinating disease in humans. It is a chronic disease with complex etiology. It is characterized by the presence of multifocal plaques of demyelination in brain that eventually lead to motor and sensory disorders. Many studies have described association between HHV-6 and MS. It was reported that HHV-6 antigens could be detected only in oligodendrocytes in the plaque regions of the brain but not in unaffected regions of the brain [360]. Brains from healthy persons were also found negative for these antigens in this study. Using virus-specific monoclonal antibodies, the researchers found reactivity with oligodendrocytes (ODC) in the affected areas of the brain. The reactivity was more prominent in the nuclei of these cells [360]. It is noteworthy that oligodendrocytes are the cells that produce myeline, which wraps around axons of neurons. Lack of myelination around axons may cause poor conductance of nerve impulses. As stated earlier, HHV-6 has been shown to infect both ODC and their precursor glial cells *in vitro* and affect their function [268]. A more convincing case for the association of HHV-6 with MS was made by Soldan et al [361]. The worker found that the titers of IgM antibodies against an early antigen of HHV-6 (p41/38) become significantly elevated in MS patients as compared to the control subjects. These antibodies were also found more frequently in MS patients: 73% of MS patients were positive as compared to 18% of the control subjects. The viral proteins as well as transcripts were also found in the plaque lesions. Since the presence of virus-specific IgM antibodies represents an ongoing viral infection, these data strongly suggest that MS patients certainly are experiencing HHV-6 infections differently as compared to the control subjects. The workers also reported that a higher %age of the MS patients (66% vs 33%) showed lyphoproliferative reponses to HHV-6A as compared to control subjects. Both groups, MS patients and control individuals responded equally to HHV-6B and HHV-7 [362]. Interestingly increased levels of soluble CD46, which is considered as a

cellular receptor for HHV-6, were also present in MS patients [363]. Moreover, U94-specific ELISA revealed significant difference between MS and control subjects: more antibody prevalence (87% vs 43.9%) and higher titers (1:515 vs 1: 190) [364]. The HHV-6 Early and Late gene transcripts have been demonstrated in the ODC in the brains of the MS patients in postmortem studies using FISH. The ODC from the brain with MS lesions as well as from normal appearing white matter were positive. The cells were also positive for the corresponding viral proteins and viral DNA [365].

The viral DNA has also been detected more frequently from the MS plaques than from normal brains [366]. It could also be easily detected by PCR in the CSF and brains of the MS patients. Care should be taken in interpreting these data since the viral DNA could also be detected in the brains of healthy persons [366,367].

Collectively these data suggest that MS patients may be exposed to HHV-6 differently. It is believed that early childhood infections with the virus establish themselves in CNS as persistent rather than latent infections. Later in life viral reactivation occurs in oligodendrocytes (ODC). This induces local immune responses in the CNS causing the development of plaques and clinical manifestation of MS. The virus damages ODC directly and/or indirectly via host responses. Consequently the ODC cannot produce myelin to wrap around axons resulting in poor conduction of nerve impulses. Despite strong evidence of an association between HHV-6 and MS, there is no direct proof that the virus causes MS. It has also been argued that HHV-6 reactivations may result from disease-related pathological processes [251]. Furthermore, it is also argued that MS causes a breach in the blood-brain barrier and this may allow the virus to reach and localize in the brains of the patients. In that case, the presence of the virus may represent a symptom of the disease. The effects of antiviral treatment on the course of the disease, if any, are not known. Continuous use of immunosuppressive drugs like natalizumab (a humanized anti-α4 integrin, which decreases immune cells in the blood and CSF) in these patients may further activate viruses like HHV-6 and JCV and cause progressive multifocal leukoencephalopathy [359].

MALIGNANCY

HHV-6 has been reported to act as a factor in the development of several malignant disorders. However, no definitive associations have been proved (reviewed in [3]). These malignancies include Hodgkin's disease, non-Hodgkin's lymphoma, Acute Lymphoblastic Leukemia (ALL), oral and cervical carcinomas [368-371]. HHV-6B DNA is present in in about 6.8 % bladder carcinoma samples [372]. Increased titers of the virus-specific antibodies are found in the sera of patients suffering from leukemia and lymphomas. Furthermore, viral DNA could also be detected from the tissues of the patients of these malignancies [369,370]. In vitro, HHV-6 DNA fragments have been shown to transform human keratinocytes [285]. The viral gene DR7 (or ORF-1) from the viral strain U1102 encodes a protein that can bind and inactivate the tumor suppressor p53. The DR7 protein

binds p53 at the DNA-binding face and sequesters it into cytoplasm[30]. DR7 sequences have been found in several angioimmunoblastic lymphadenopathiesm, Hodgkin's and non-Hodgkin's lymphomas, and glioblastomas [30]. As mentioned earlier, another viral late protein, the product of the U14 orf, can also bind and inactivate p53 [205]. It is noteworthy that p53 inactivation could predispose cells to genetic lesions and carcinogenesis.

In one study no significant difference was found in the prevalence of HHV-6-specific antibodies between leukemic children and non-leukemic control children [373]. HHV-6 can infect human cervical epithelial cells harboring latent HPV virus and increase HPV mRNA expression. The HHV-6 UL16 protein (pZVB-70) and a genomic clone pZVH-14 (containing U30) transactivated transforming genes of HVP. However, no associations between HHV-6 infection and HPV-induced neoplasia could be observed in clinical studies [374,375]. HHV-6 can enhance oncogenic potential of EBV by increasing expression of LMP-1 in EBV genome-positive Burkitt's lymphoma cells [376]. It is noteworthy that LMP-1 is an EBV-encoded oncoprotein. The role of EBV is more important than HHV-6 in inducing HIV-related malignancy. For example in one study, EBV (50 genomes per 10^5 PBMC in children with more than 200 CD4+ T cells per cmm) but not HHV-6 was found to be a risk factor for developing malignancy in HIV-infected children [377].

It is tempting to speculate that U94 protein may have blunted the pro-malignancy ability of HHV-6. The protein effectively acts as a tumor suppressor.

INFECTIOUS MONONUCLEOSIS

Infectious mononucleosis or glandular fever usually occurs in teen-agers in developed countries. It is a self-limiting mild lymphoprolifrative disorder accompanied by fever, pharyngitis and occasionally with neurological manifestations. Most of the cases occur when primary infections with EBV and/or HCMV occur in adolescence rather than in childhood. HHV-6 has also been reported as an etiological agent of infectious mononucleosis and lymphadenopathy [378]. It was reported that 8 out of 27 (30%) patients show serological evidence of infection with HHV-6 but not with EBV or HCMV and are negative for heterophile antibody. However the authors could not distinguish whether it was caused by primary or reactivated HHV-6 [379]. It is noteworthy that HHV-6 and EBV are frequently detected in human tissues. *In vitro* studies have shown that the two viruses mutually activate each other (detailed below in the section on Effects on other Viral Infections). Thus the two viruses may contribute towards the pathology of this syndrome.

DRUG HYPERSENSITIVITY SYNDROME

Viral infections could play a role in drug-induced hypersensitivity syndrome. HHV-6 has been suggested to play a role in this syndrome [380,381]. The syndrome is characterized by eosinophilia, mononucleosis-like systemic symptoms, skin eruption, hepatic dysfunction, lymphadenopathy and leukocytosis with the presence of atypical cells.

The syndrome may occur with the use of a variety of drugs including ibuprufen, allopurinol, sulfasalazine, phenobarbital or carbamazepine [380,382,383]. Descamps et al have described 7 patients suffering from the syndrome; 4 of the patients developed high titers of IgM against HHV-6 and two seroconverted for anti-HHV-6 IgG. These findings suggested a primary infection with HHV-6 in these patients. However, viral DNA could not be detected in the serum samples collected in the early phase of the infection. In susceptible patients, the consequences of the virus-induced drug hypersensitivity syndrome could lead to grave consequences [380].

SKIN ERUPTIONS

Gloves and Socks Syndrome: The virus is associated with the etiology of so-called "Gloves and Socks" syndrome. The skin disease is characterized by the appearance of papular-purpuric lesions on hands and feet. It is an acute benign disease [384].

Gianotti-Crosti Syndrome: The virus has also been implicated in the etiology of Gianotti-Crosti syndrome. The syndrome was reported in an 8-month old child with characteristic monomorphic papules on his/her cheeks, buttocks and extremities [385]. The eruptions were followed by roseola. Serological studies confirmed the presence of primary infection with HHV-6. Another study reported two young children diagnosed as having Gianotti-Crosti syndrome. By PCR, the children were found having active infection with HHV-6 [386].

Ptyriasis Rosea: It is a condition of benign dermatosis. The association of HHV-6 with the condition is controversial. In a case control study, no evidence could be found for a recent infection with HHV-6 in this syndrome [387]. However, later studies have found evidence of systemic infections with HHV-6 and HHV-7 in the patients diagnosed with Ptyriasis Rosea [388].

Thrombocytopenic Purpura: It is a skin complication seen in young children recovering from acute infection with HHV-6. Other complications may include fulminant hepatitis with liver failure and fatal myocarditis [389-392].

Myocarditis: Prezioso et al have reported a case of an immunocompetent 13-month old child with disseminated systemic infection with HHV-6. The child died of cardiac arrest. The autopsy revealed the presence of intranuclear viral inclusion bodies in different organs including heart. The damage of parenchymal tissue was evident in the organs [252]. Since then several cases of HHV-6-induced myocarditis have been reported in children. Recently, a case of diffuse myocarditis and fulminant hepatitis with systemic HHV-6B reactivation and fatal outcome has been described [393].

Autism

In a recent study, a significant association of HHV-6 infection with autism was demonstrated [394]. Of the 48 blood samples from the patients with autistic spectrum disorder, 58.3% were positive for Mycoplasma compared with 4.7% normals (OR 13.8; $p<0.001$), 8.3% positive for

Chlamydia pneumoniae (OR 5.6; p<0.01) and 29.2% for HHV6 infection (OR 4.5; p< 0.01). Further studies are needed to confirm these findings.

H. EFFECTS ON OTHER VIRAL INFECTIONS

Humans are often simultaneously infected with HHV-6 and other viruses like HHV-7, EBV, HCMV, and HCV etc. Co-infections are likely to be more pathogenic for the host. In this regard it was recently shown that a single human cell could be simultaneously infected with HHV-6, HIV-1 and HCV, and the infected cell dies more quickly [395]. HHV-6 may affect the natural course of other viral infections. It induces expression of CR2, the receptor for EBV, and increases susceptibility of human cells to infection with EBV [396]. It has been demonstrated that HHV-6 stimulates the lytic cycle of replication in cells latently infected with HHV-8 [397]. It was also shown that the virus induces lytic infection of EBV by activating Zebra promoter in latently infected B cells. It transactivates the promoter by inducing increased binding of CREB to a c-AMP response element (CRE) present in the promoter region [398]. In another study, variant A, but not B, of HHV-6 was shown to infect EBV-positive lymphoid cells and increase expression of EBV's early genes BZLF1 and BMRF1 [399]. The virus also increases expression of the latent membrane protein-1 in EBV-positive Burkitt'lymphoma cells [376]. These data suggest that HHV-6 co-infection may enhance oncogenic potential of EBV. It is noteworthy that EBV-infected B cells become more susceptible to infection with HHV-6. The anti-HHV-6 antibody titers are often higher in the patients co-infected with EBV or HCMV [400].

HHV-6 does not seem to affect the course of HCV infection in humans although the two viruses have been shown to co-infect humans *in vivo* as well as T cells in *in vitro* studies [395]. However, co-infection with the two viruses increases risk to develop fibrosis. HHV-6 may also complicate HBV-induced hepatitis and alcoholic liver disease by augmenting autoantibody formation [401]. The virus also infects and arrests ATL. This results in the outgrowth of uninfected cells [402]. HHV-6 can infect epithelial cells and upregulate E6 and E7 oncoproteins in cervical epithelia cells [114]. Thus, the virus may enhance HPV-induced cervical tumors.

I. OTHER DISEASES:

The virus has also been associated with Bell's paralysis, Kikuchi's Necrosing Lymphadenitis, and Chronic Fatigue Syndrome [143,403-406].

TREATMENT:

The primary infections in children are usually self-limiting and do not require any antiviral treatment. However, treatment may be needed in the case of complications or as prophylaxis in immunosuppressed persons against reactivations. Unfortunately, no controlled clinical trials have been conducted to test the efficacy of drugs against HHV-6. *In*

vitro studies have shown that ganciclovir, foscarnet and cidofovir effectively inhibit HHV-6 replication [407].

Ganciclovir and acyclovir are acyclic nucleoside analogs and target viral DNA polymerase. Their triphosphate metabolites inhibit the enzyme by competing with natural substrate dGTP, form dead end complex due to absence of a –OH group in the acyclic chain and cause termination of DNA synthesis (reviewed in [408,409]). The selective antiviral effects of these drugs are due to the fact that Herpesviral DNA polymerase has higher affinity for the triphosphate metabolites than the cellular DNA polymerase. More importantly a viral kinase (encoded by α- and γ-Herpesviruses) phosphorylates these drugs into active metabolites. In the case of β-Herpesviruses, a virus-produced phosphotransferase (pUL97) converts ganciclovir into monophosphate. In the herpesvirus-infected tissues acyclovir is converted into a reverse transcriptase inhibitor [410]. Two cellular enzymes deoxyguanylate (dGMP) kinase and nucleoside diphosphate (NDP) kinase convert monophosphate into active metabolite. The HHV-6 gene U69 encodes the phosphotransferase. However, it is ten fold less efficient in phosphorylating ganciclovir than its HCMV homolog UL97 [411]. It is noteworthy that UL97 homologs exist in all human herpesviruses and they include U13 of HSV-1, Orf 47 of VZV, BGLF4 of EBV and ORF 36 of HHV-8 (reviewed in [412]). The exact role of U69 in the replication of HHV-6 remains unknown. Its HCMV homolog UL97 binds DNA polymerase processivity factor and plays an important role in viral replication by facilitating the nuclear egress of viral capsids. It is a serine/threonine kinase, can phosphorylate itself as well as UL44, pRb and ganciclovir. The phosphorylation of pRB prevents formation of aggresomes [76,77,413,414]. Acyclovir is not effective against this virus in *in vitro* studies [415]. In the case of ganciclovir, B variants are more sensitive than A variants [416]. Some studies have demonstrated that antiviral treatment against HHV-6 is beneficial in BMT recipients suffering from encephalitis and/or myelosuppression [242,267,417,418]. However, in some cases of fulminant infection, no response was observed with the therapy [321,419]. It has also been shown that prophylactic treatment with ganciclovir in children receiving BMT from allogeneic donors significantly decreases the incidence of HHV-6 infections. The treated children not only show a decreased viral load but also improved clinical condition [276,420]. Thus the patients with serious HHV-6 infections are very likely to benefit from antiviral therapy. Early HHV6 treatment in the transplantation setting may improve clinical outcomes. Randomized placebo controlled clinical trials are lacking. Non-randomized studies in HSCT recipients suggest that prophylactic gancyclovir treatment could prevent HHV6 reactivations [276,420]. Foscarnet and ganciclovir in combination or alone have been recommended for HHV6-related neurological disease by the International Herpes Management Forum [421].

Most of the classical drugs used against Herpesviruses have important drawbacks[422]. Ganciclovir has low bioavailabilty upon oral administration. This may necessitate intravenous administration. The valyl ester of the drug, valganciclovir, is much better in this respect.

Therefore, new strategies for the treatment of these infections should be considered.

It is noteworthy that antiviral chemotherapeutics have their own toxic effects. Each agent may cause one or prominent effects. For example, ganciclovir has mainly myelotoxic effects and may cause irreversible neutropenia and thrombocytopenia [423]. Lowering the dose poses the risk of emergence of drug resistant viruses.

Foscarnet (phosphonoformic acid) is a pyrophosphate analog. It binds reversibly to DNA polymerase close to the pyrophosphate-binding site, and requires no prior activation by viral or cellular enzymes. It has higher affinity for viral DNA polymerase than the cellular enzyme. The drug has broad spectrum, being effective against all Herpesviruses. The drug is relatively less toxic even to continuously growing human cells *in vitro*. However, *in vivo* the drug causes dose-dependent nephrotoxicity. It is used mainly for treatment of HCMV-induced retinitis in AIDS patients. Mixed results have been obtained with Foscarnet in HHV-6 encephalitis in transplant recipients [317,321,419].

Little data is available concerning the comparative efficacy of ganciclovir and foscarnet for treatment of HHV-6. Therefore the choice of the antiviral agent may depend upon the clinical symptoms of the infection in each patient. These agents should be used very judiciously because a prolonged or intermittent exposure to suboptimal doses may select for resistant strains of the virus. It was observed that treatment with ganciclovir or foscarnet decreases viral load in the same fashion as for HCMV [346].

Cidofovir is an acyclic nucleoside phosphonate analog of dCMP. Its inhibitory effects are not restricted to Herpesviruses; all DNA viruses are susceptible to this drug. No viral enzyme is needed to convert Cidofovir into active metabolite. Several cellular enzymes can convert it into active diphosphate metabolite [424]. Higher affinity of viral DNA polymerases than their cellular counterparts explains selective inhibitory effect of this drug for the viruses. Its incorporation into a growing molecule of DNA does not result in chain termination. The DNA containing Cidofovir diphosphate cannot be duplicated [420,425]. The drug is recommended for the treatment HCMV-induced retinitis in AIDS patients. The drug is highly nephrotoxic. Despite being highly effective in inhibiting HHV-6 replication *in vitro,* alone and in combination with Foscarnet, the drug is used only in cases that do not respond to Ganciclovir or Foscarnet. Nevertheless in the case of an HHV-6-induced encephalitis in an immunocompetent individual, Ganciclovir proved more effective and safer than Cidofovir [426].

In addition to these drugs, arylsulfone derivatives have been described as new antiviral agents for β-Herpesviruses, especially for HHV-6 and 7 [427]. These componuds inhibit viral DNA synthesis in 95% of the virus-infected cells by inhibiting expression of late genes in the infected cells.

Small interfering (si)RNA hold great promise as antiviral agents if proper technologies could be developed for their delivery to in vivo situations. In the case of HHV-6, small interfering (si)RNA targeted to the U38 gene, which encodes viral DNA ploymerase, inhibits HIV-B replication

in Sup-T1 cells[428]. Further viral genes could be tested as potential targets for inhibiting viral replication.

VACCINATION

No effective vaccine is yet available against HHV-6. In fact, Varicella-Zoster is the only Herpesvirus for which an effective vaccine exists and is used.

DRUG RESISTANCE

The emergence of drug-resistant strains occurs when sub-optimal doses of drugs are used for long periods of time. Immunosuppressive patients usually receive prophylactic treatment with acyclovir or ganciclovir against reactivation of HSV-1 and HCMV. No treatment has been formally approved for HHV-6 infection. However, the treatment with ganciclovir would also work against HHV-6 [276,420]. It has been well documented that both HCMV and HSV-1 can develop resistance against these drugs. The resistance occurs due to mutations in the viral phosphotranferase (U97) and viral DNA polymerase genes. Such mutations could also develop in the U69 and polymerase genes of HHV-6, but this aspect of the virus has not been studied extensively. Ganciclovir-resistant viruses developed *in vitro* showed mutations in U69 gene resulting in the amino acid change (M318V) in the protein and a mutation in the U38 gene causing (A961V) change in the amino acid sequence of DNA polymerase [429]. The U69 gene encodes a protein kinase that phosphorylates ganciclovir and converts it into its pharmacologically active metabolite. The M318V was reported in the HHV-6 virus infecting PBMC in an AIDS patient, who had received long-term treatment with ganciclovir [429]. It was demonstrated that introduction of mutations in the codons 318, 448, and 465 in the U69 gene of HHV-6 impaire the ability of U69 kinase to phosphorylate ganciclovir in a Baculovirus system [430]. The mutations were analogous to those documented for HCMV in the homologous genes. The ganciclovir-resistant HHV-6 also shows cross-resistance to cidofovir.

PERSPECTIVE

HHV-6 is a ubiquitous virus, which usually establishes life-long latent infections in a variety of tissues in human body. The virus has enormous capacity to become reactivated and cause a vast array of diseases and clinical conditions in the immunocompromised host. Attempts should be made to understand the conditions that regulate viral latency, persistence and reactivations.

Although HHV-6 has been associated with many disease conditions in humans, there is no direct proof that the virus is behind etiology of these diseases. The ubiquitous nature of the virus makes it very difficult to

draw definite conclusions. It would be highly desirable to develop animal models of the infection. Although chimpanzees and Rhesus monkeys could be infected with the virus, these animal models are too expensive and many ethical considerations preclude their use in experimentations with the virus.

An important question that needs to be addressed is whether it is the infection or it is the virus-specific inflammatory and immune response that triggers many clinical conditions with which the virus has been associated. The answers may have important implications for treating these conditions.

A great effort is made in understanding the pathobiology of HHV-6. In this regard, research is more focused on understanding functions of various viral genes. It is increasingly being realized that non-protein encoding micro (mi)RNAs play an important role in regulating expression of genes in humans as well as viruses (reviewed in [431]). Other herpesviruses including HCMV, which is genetically closely related to HHV-6, encode their own miRNAs to regulate gene expression profiles of the host cells. However, little is known about HHV-6. The viral genome has the potential to encode several miRNAs. Validating these miRNAs and discovering their (viral and/or cellular) RNAs could provide novel insights about immunobiology of the virus. This should be an area of high priority research.

HHV-6 has two distinct variant types, which differ in epidemiology, antigenicity, tropism, pathogenicity and disease spectrum. Present diagnostic tests are not very efficient in distinguishing between variant-specific latent and active infections. The development of such tests is a prerequisite for a better understanding of the role of A and B variant types in different HHV-6-associted clinical conditions and syndromes.

ABBREVIATIONS

AIDS: Acronym for Acquired immunodeficiency syndrome, BMT: Bone marrow transplant, CBL: Cord blood lymphocytes, CBMC: Cord blood mononuclear cells, CFS: Chronic Fatigue Syndrome, CNS: Central nervous system, CSF: Cerebrospinal fluid, DC-SIGN: Dendritic cell-specific itegrin-grabbing non-integrin molecule (CD 209), DR: Direct Repeat, E: Early (genes), EBV: Epstein-Barr virus, ER: Emergency room, FISH: Fluorescent in-situ hybridization, GRIM-19: Gene associated with Retinoic acid and Interferon-induced Mortality-19, GvHD: Graft versus host disease, HBLV: Human B Lymphotropic Virus, HBV: Hepatitis B virus, HCV: Hepatitis C virus, HCMV: Human Cytomegalovirus, HIV: Human Immunodeficiency virus, HSCT: Hematopoietic stem cell transplant, ICAM: Intercellular cell adhesion molecule, IE: Immediate Early (genes), L: Late (genes), LTR: Long terminal repeat, ManHV: Mandrill Herpesvirus, mCRP: Membrane-bound complement regulatory protein, MRI: Magnetic resonance images, MS: Multiple sclerosis, ODC: Oligodendrocytes, ORF: Open reading frame, PanHV: Pantrogylus or Chimpanzee Herpesvirus, PBMC: Peripheral blood mononuclear cells, PBL: Peripheral blood lymphocytes, PCR: Polymerase chain reaction, qPCR: Quantitative PCR, RANTES:

Regulated upon activation, normal T cell expressed and secreted (CCL-5), RT-PCR: Reverse Transcriptase-PCR, SCR: Short consensus repeat, SCT: Stem cell transplantation, SOT: Solid organ transplantees.

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Table 1. Human Herpesviruses

Generic Name	Common Name	Disease	Year Discovered (Ref)
αHerpesviridae:			
HHV-1	Herpes Simplex virus type 1 (HSV-1)	Fever, Muco-cutaneous blisters Encephalitis	1873 (1)
HHV-2	Herpes Simplex virus type 2 (HSV-2)	Genital sores, Encephalitis	1873 (1)
HHV-3	Varicella Zoster Virus (VZV)	Chicken pox, Shingles or Zoster (upon reactivation)	1952 (2)
β Herpesviridae:			
HHV-5	Human Cytomegalovirus (HCMV)	IM, Retinitis, Pneumonitis Transplant rejection; Multiorgan diseases PTLD, atherosclerosis, restinosis	1904 (3)
HHV-6	Human Herpesvirus-6 (HHV-6)	Exanthum subitum (Roseola), IM,PTLD	1986 (4)
HHV-7	HHV-7	ES, IM,	1990 (5)
γ-Herpesviridae			
HHV-4	Estein-Barr virus (EBV)	IM, XLPD, BL, Gastric carcinoma,PTLD	1964 (6)
HHV-8	Kaposi Sarcoma Herpesvirus,(KSHV)	Kaposi' Sarcoma, MM, PEL	1994 (7)

Congenital infections with HCMV may cause hearing loss, mental retardation; its reactivations in AIDS patients and transplantees cause retinitis, pneumonitis, generalized infection, atherosclerosis and restenosis. BL: Burkitt's lymphoma; ES: Exanthum subitum; IM: Infectious mononucleosis; MM: Multiple Myeloma; PEL: Peritoneal effuse lymphoma; PTLD: Post-Transplant Lymphoproliferative Disease; XLPD: X-linked Proliferative Disease.

Table 2. Differential characteristics of A and B variants of HHV-6

Characteristic	**A variants**	**B variants**
Tissue Tropism	More neurotropic	Tropism for salivary glands, lymph nodes, skin and tissues
U83 (a virokine)	Not secreted	Secreted
Disease spectrum	Encephalopathy, CFS	Primary infections (Roseola)
Permissive Cell lines*	HSB-2 (GS-1) JJHAN (U1102)	MOLT-3, MOLT-4
Prevalence	Brain, CSF	Saliva, Blood, PBMC
Induces fusion from without	Efficiently	Poorly**
Diseases	Unknown	Subitum Exanthum, Pitrysia rosea
P53 induction	Very strong	Relatively mild
Orf	110	119
Induction of apoptosis	Strong	Mild

*Despite their differential ability to grow in cell lines, both the variants grow efficiently in IL-2 activated cord blood lymphoctes. HHV-6A are recovered mostly from immunocompromised than immunocompetent persons and are not associated with any specific disease.
** Except for PL1
CFS: Chronic Fatigue Syndrome, CSF: Cerebrospinal fluid.

Table 3. Isolation of A and B variants from different human organs and disease conditions

	A	B
Roseola	+/-	++++
MS	++	++
Lymphomas, tumors	++	++
Reactiv in Transp	++	++
AIDS	++	++
Peripheral blood	+	++++
Salivary gland	-	+++++
Skin	++	++++
Brain	++	++
Lymph nodea	-	++++
Other tissues	-	++++
Healthy serum	-	-
Roseola serum	-	++++
Serum from AIDS CFS, Lymphomas	+++	+
Saliva	-	++++
CSF (children)	++++	+

The + and – signs refer to the presence and absence of the variant, respectively. Increased numbers of + signs denote increased frequency of the variant.

Table 4. Divergent genes between HHV-6 (A and B variants) and other Herpesviruses

Orf	Product	HHV-6 A	HHV-6 B	Other Herpesviruses
U39-U42	gpB (fusin) transactivator	α α	α α	γ 1 α or β
U73	OBP	α	α	β
U89-90	IE-A	α	α	α
U94	Rep	α	α	absent
U16-17	IE-B SS	β	α	α
U91	IE-A (anti-sense), NS	α	B	Absent
	IE-A (anti-sense), SS	β	α	absent
U18-20	IE-B	β (526 bp; no U19)	γ (1900 bp with U19)	α
U19	IE-B	β	β	α
U31	LTG	β	β	γ
U41	MDBP	β	β	β
U53	Protease	β	β	γ
U 97-100	gp82/105	γ		
U82	gL	γ, (transcript under beta but spliced under γ)		
U48	gH	γ		
U100	gQ, Q2 (gp 85/105)	γ		
U47	gO	γ		

DBP: Major DNA binding protein; OBP: Origin binding protein; LTG: Large tegument glycoprotein; NS: Non-spliced; SS: Singly spliced; DS, double spliced.
Compiled from the reference [26]

Table 5. Functions of some important HHV-6 genes:

Orf-1	IE/E/L	Also called DR-7; Oncogene; Encoded protein binds and inactivated p53 Transactivation of viral and cellular promoters
U3	IE	Transactivation; a tegument protein
U11	L	The major viral structural protein (p100); varies between A and B variants
U12	L	Viral chemokine receptor
U14	L	p53-binding protein, inactivates p53 and recruits it into virions
U16-19	IE	IE-B regulatory proteins
U18	L	gpM
U19	IE	
U21		Binds MHC class molecules and degrades them
U22	L	gp22 (A unique glycoprotein of HHV-6)
U24		Interferes with endocytic recycling pathway; downregulates CD3 and transferrin receptor on cell surface
U27	E/L	PNA polymerase processivity factor; functional homologue of PCNA
U28	E	A sub-unit (R1) of the viral ribonucleotide reductase
U31	IE	Large tegument protein
U38	E	DNA polymerase
U39	E	gpB, role in viral fusion and infection
U41	IE	Major DNA binding protein
U42	IE	Transactivator; DPPF, a homologue of human PCNA
U43/74/77	E	Helicase-primase complex
U45	E	The viral dUTPase
U46	?	gpN
U47	L	gpO
U48	L	gpH; role in binding to the viral receptor
U51	E	Decoy chemokine receptor (like an opioid receptor)
U53	E	Protease
U54	E	Homologue of the HCMV UL42, encodes a tegument protein
U57	E	Major capsid protein
U64	L	The viral packaging protein
U69	L	A serine threonine kinase, localizes to the nucleus, imparts sensitivity to ……..Ganciclovir
U72	L	gpM; a conserved herpesviral glycoprotein
U73	IE	Origin binding protein
U79/80	IE	36, 41, 44, 59 kD nuclear proteins Viral DNA replication
U81	E	The viral Uracil DNA glycosylase
U82	L	gpL; a part of the glycoprotein complex that binds the viral receptor
U83	E/L	A virokine, modulates chemotaxis of monocytes (Unique to HHV-6)
U90/U86-89	IE	IE-A regulatory proteins
U94	IE	Regulatory Rep protein, maintains latent infection, inhibits angiogenesis and lymphomagenesis; a role in viral integration?
U95	IE	A member of the US22 gene family; Interacts with GRIM-19; Transactivation
U100	L	gpQ1-Q2 or gp82-105; varies between A and B variants

HCMV: Human Cytomegalovirus; RANTES: Regulated and Normal T cell Expressed and Secreted; PCNA: Proliferating cell nuclear antigen; IE, E and L refer to Immediate Early, Early and Late genes, respectively.

Table 6. HHV-6 pirated genes

Gene	Protein Function	Homologues
U12	Viroceptor	GPCR; Decoy chemokine receptors
U27	DNA polymerase stimulator	PCNA
U51	Viroceptor	GPCR; Decoy chemokine receptors
U83 (L)	Virokine	Beta-chemokine
U22	Virokine	Chemokine
U14	p53 antagonist	MDM2 but does not degrade P53
DR7	p53 antagonist	MDM2 but does not degrade it into virion incorporates p53
U94	Repressor	Rep68/78 gene of AAV-2

Table 7. Mechanisms of HHV-6 for evading host's antiviral responses

1. By infecting immune cells latently and lytically
2. By inducing death of non-infected cells
3. By manipulating chemokine and cytokine systems of the host
4. By deregulating cell cycle control mechanisms of the host
5. By interfering with DNA damage response of the host cells
6. By modulating cell surface molecules of the host
7. By protecting cells from the complement-mediated destruction
8. By interfering with antigen presentation pathway of the host
9. By pirating host cell genes
10. By interfering with autophagic response of the host cells

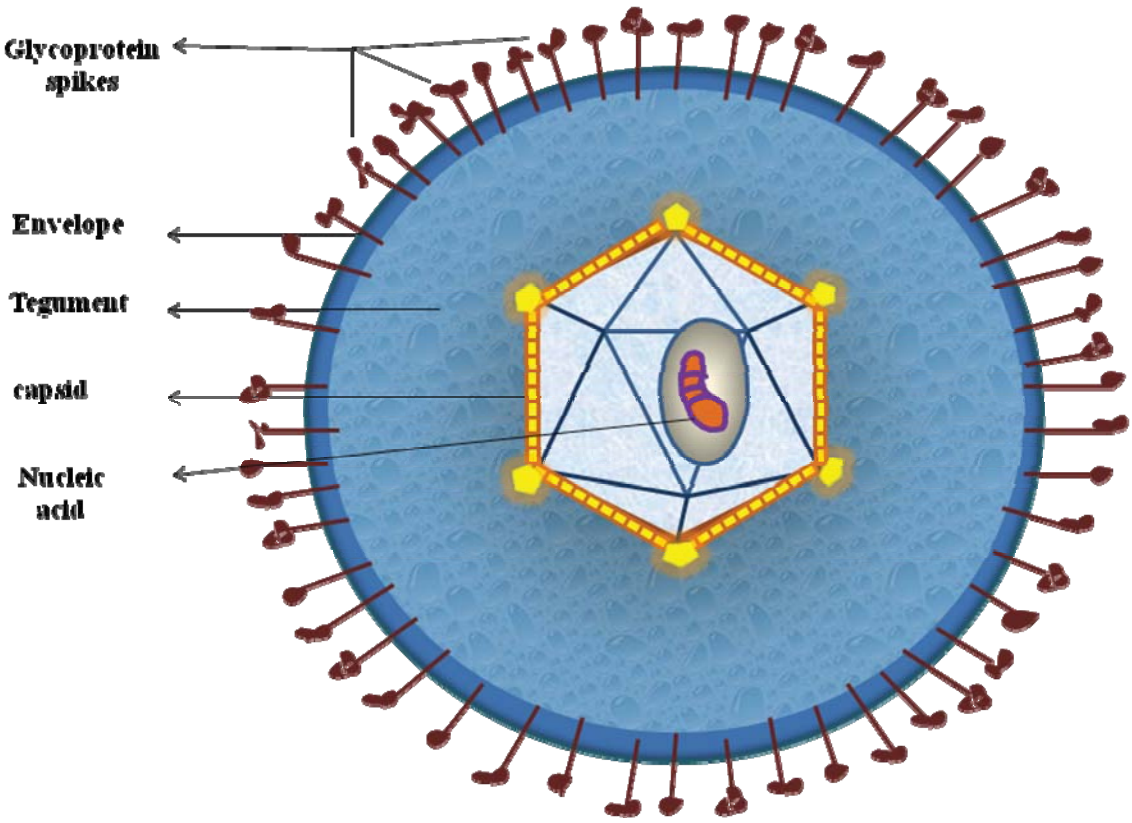
Table 8. Methods used to diagnose HHV-6

Assay	Advantages	Disadvantages
Virus isolation	Informative about the vira variant and its replication status	Labour intensive; Time consuming; Technically difficult and requires specialized infrastructure and expertise
PCR	Can be performed on plasma or PBMC Viral load can be determined; Could be variant specific; Technically easy; Works on archived samples	False negative and positive Appropriate controls and cut-off needed; Uninformative about status of viral replication
RT-PCR	Active or latent infection	Fresh samples and appropriate positive and negative controls needed
Serology (detection virus-specific antibodies)	Less time consuming; Can distinguish between primary infection and reactivation	Maternal antibodies may interfere results in diagnosing primary infection in children; Immunocompromised patients may have no antibody responses; Current assays cannot distinguish A and B variants
Antibody avidity	Can distinguish between primary and chronic infection	A specialized serology test and has the same disadvantages as described above for serology
Western blots	Can be used to detect virus-specific antibodies or proteins; Informative about viral replication and variant	Requires specialized infrastructure and expertise; Long turn-around time

PBMC: Peripheral blood mononuclear cells, RT-PCR: Reverse Transcription-Polymerase Chain Reaction.
Compiled from literature.

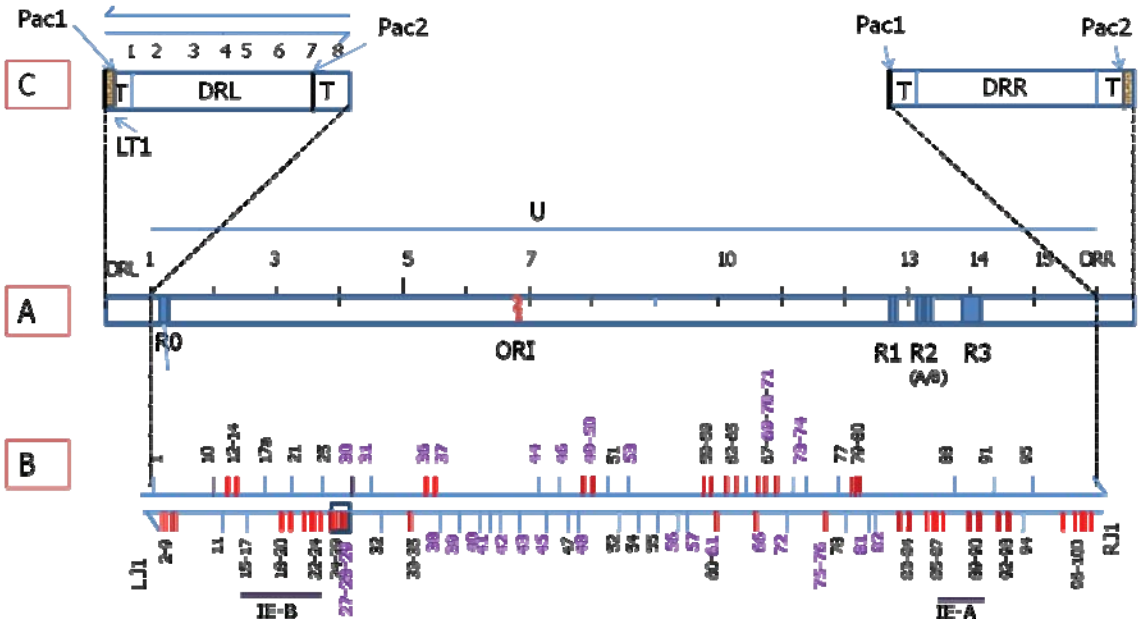
Figures:

Figure 1. Structure of a typical HHV-6 virion



In addition to the viral glycoproteins, the envelope also contains cellular proteins (not shown). See text for details.

Figure 2. Genomic organization of HHV-6

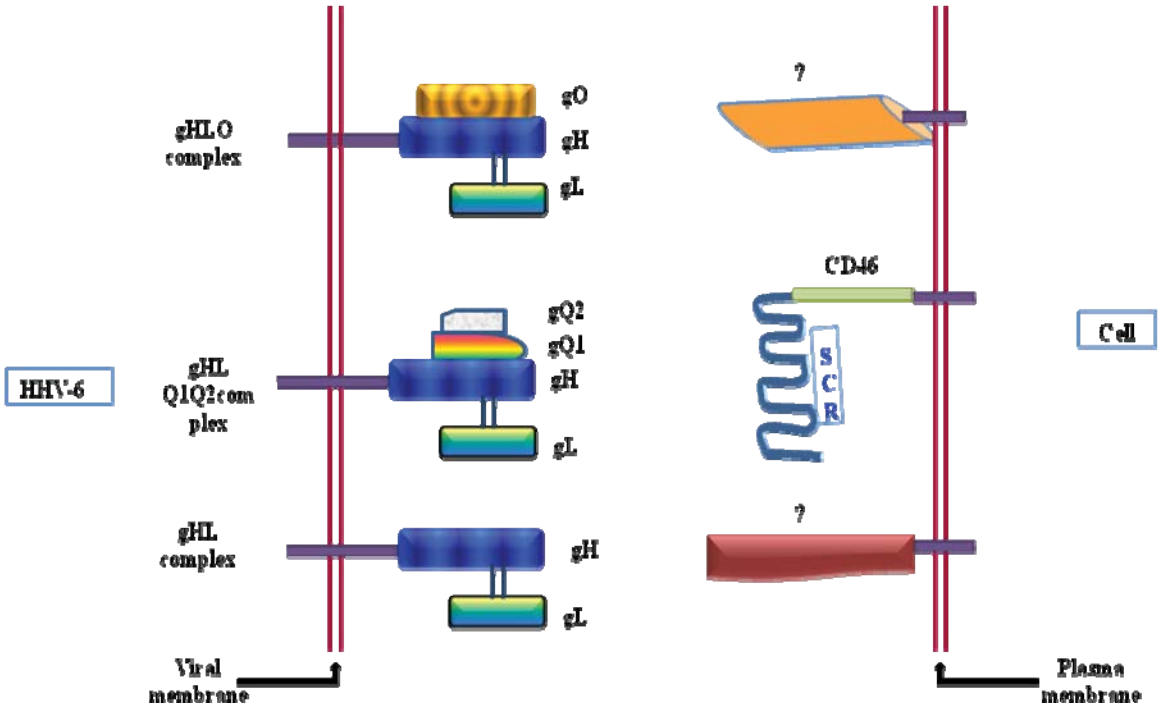


A. Organization of HHV-6 genome showing left and right direct repeats (DRL and DRR, respectively), origin of viral replication (ORI), three regions of repats (R1, R2 and R3). A/B below R2 indicates R2A and R2B regions, respectively. The numbers indicate kilo base pairs.

B. The Figure shows location of different viral genes (numbered from left to right) trascribed leftward (red) or rightward (light blue). IE-A and IE-B indicate location Immediate Early A and B genes, respectively. LT1, LJ1 and RJ1 indicate Left Terminal-1, Left Junctional-1 and Right Junctional-1 ORFs, respectrively. They span telomeric sequences.

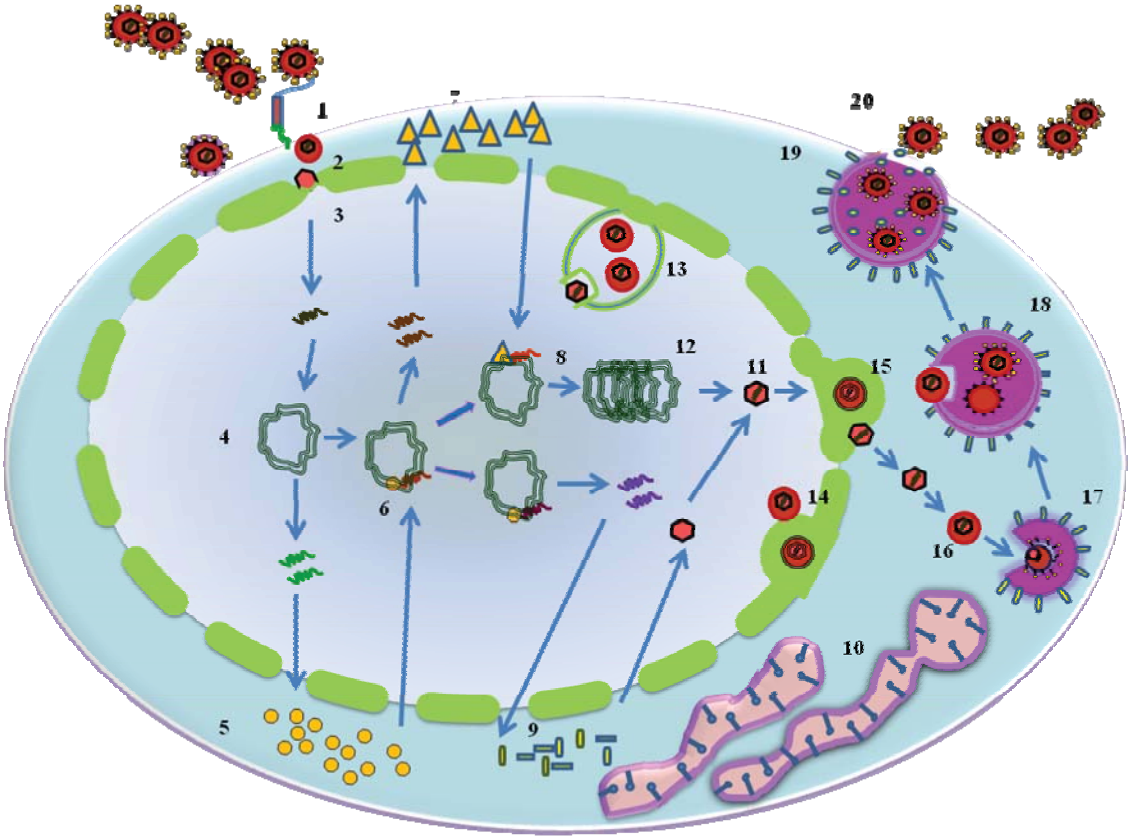
C. The Figure shows details of the Right and Left Direct Repeats. T indicates telomeric sequences. DR1-8 shown in DRL are duplicated in DRR (not shown). See text for deatails.

Figure 3. HHV-6 binding to CD46



Three glycoprotein complexe gHLQ1Q2 binds CD46. Other viral complexes (gHLO and gHL) can also mediate virion binding and infection. Their receptors on human cells remain unknown and are indicated by a question mark (?). SCR are short consensus repeats.

Figure 4. Replication Cycle of HHV-6



The cycle comprises a series of discrete steps that occur as a continuum and include:

1. The virions bind to viral receptor and the viral envelope fuses with plasma membrane of the cell releasing nucleocapsid in cytoplasm, 2. The tegument and capsid proteins engage microtubule (MT)-associated motor proteins and dock the nucleocapsid along the MT to nuclear pores, 3. The nucleocapsid opens and releases viral genome into the nucleus, 4. The genome circularizes and IE genes are transcribed, 5. IE mRNAs leave nucleus and are translated in the cytoplasm, 6. IE proteins enter nucleus and activate transcription of E genes, 7. Early gene mRNAs leave nucleus and are translated in the cytoplasm, 8. Early proteins enter nucleus, transcribe Late genes, and replicate viral DNA into a long concatemer, 9. The Late gene mRNAs leave nucleus and are translated into Late proteins, 10. Some Late proteins enter nucleus while others enter into the ER and Golgi, and insert themselves into plasma membranes at sites of second envelopment of the capsids, the envelope proteins accumulate in membranes of long tube-like extensions from ER and trans-Golgi called annulate lamellae, 11. Early and Late proteins assemble into capsids, 12. Replicated viral genome concatemer is cut into unit lengths and are packaged to capsids, 13. The capsids acquire some tegument proteins in tegusomes that are derived from the inner nuclear membrane, 14. The capsids with tegument bud into inner nuclear membrane into perinuclear space, and acquire

primary envelope, 15. The primary virions bud through outer nuclear membrane into cytoplasm losing primary envelope, 16. The capsids acquire some tegument proteins that direct them to the site of secondary envelopment, 17. The capsids bud into vesicles derived from the trans-Golgi Network (TGN) and are studded with the viral envelope glycoproteins and acquire the secondary or final envelope, 18. Many capsids bud into single vesicle, 19. Many secondary vesicles bud into the primary vesicle acquiring the characteristics of a multivesicular body (MVB), 20. The mature MVB fuses with the plasma membrane releasing virions and internal vesicles (exosomes) into the exterior of the cell.

1.2 Le Cycle Cellulaire

On désigne par un cycle de division cellulaire (cdc) ou tout simplement par cycle cellulaire, un cycle complet au cours duquel une cellule se divise en deux cellules filles. La progression du cycle cellulaire est régulée par plusieurs gènes nommés cdc, tel que la cdc2.

1.2.1 Les Différentes Phases du Cycle Cellulaire

Quatre phases distinctes divisent arbitrairement un cycle cellulaire eucaryotique. Ces phases sont : G1, S, G2 et M (Figure 1). Une cellule en division passe par ces quatre phases dans un ordre précis. G1, S et G2 sont également appelées collectivement interphases. La phase M est divisée en prophase, prométaphase, métaphase, anaphase et télophase (41); revue dans (42). Le temps nécessaire pour compléter un cycle de division cellulaire dépend du type cellulaire. La division de certaines cellules peut être assez rapide et ne dure que 6 à 8 heures comme il est le cas des cellules cancéreuses. Par contre, la division des lymphocytes humains normaux est habituellement de 18-24 heures. La durée de chaque phase du cycle cellulaire dépend aussi du type cellulaire. Le passage d'une phase à l'autre du cycle cellulaire est appelé transition. Durant la progression du cycle cellulaire, G1/S et G2/M constituent deux transitions importantes (43). Au cours d'un cycle cellulaire, la cellule va synthétiser et dupliquer essentiellement son ADN (c'est la synthèse ou la phase S). Cette phase est suivie par une répartition équitable de l'ADN dupliquée chez les deux cellules filles (c'est la mitose ou phase M). Dans une cellule eucaryote typique, la phase S peut prendre de 10 à 12 heures alors que la phase M peut ne durer que quelques minutes. Ces deux processus sont séparés par deux intervalles appelés phases (Gap ou G1 et G2), durant chacun desquels, les cellules sont métaboliquement actives et elles se préparent pour la phase suivante. Au cours de la phase G1, les cellules s'engagent dans la prolifération et se préparent à la synthèse de l'ADN. Après la synthèse d'ADN à la phase S, les cellules vont se développer durant la phase G2 de telle manière que le nombre d'organelles cellulaires ainsi que la taille augmentent pour qu'ils se répartissent ensuite chez les deux cellules filles. Il est à noter que, durant un cycle cellulaire, les cellules dupliquent et divisent aussi bien leurs ADN que les organelles et les macromolécules. Les cellules au repos ou qui ne se divisent pas sont dites en phase G0 ou quiescentes. Les cellules en culture ne sont pas toutes dans la même phase du cycle cellulaire et elles sont dites

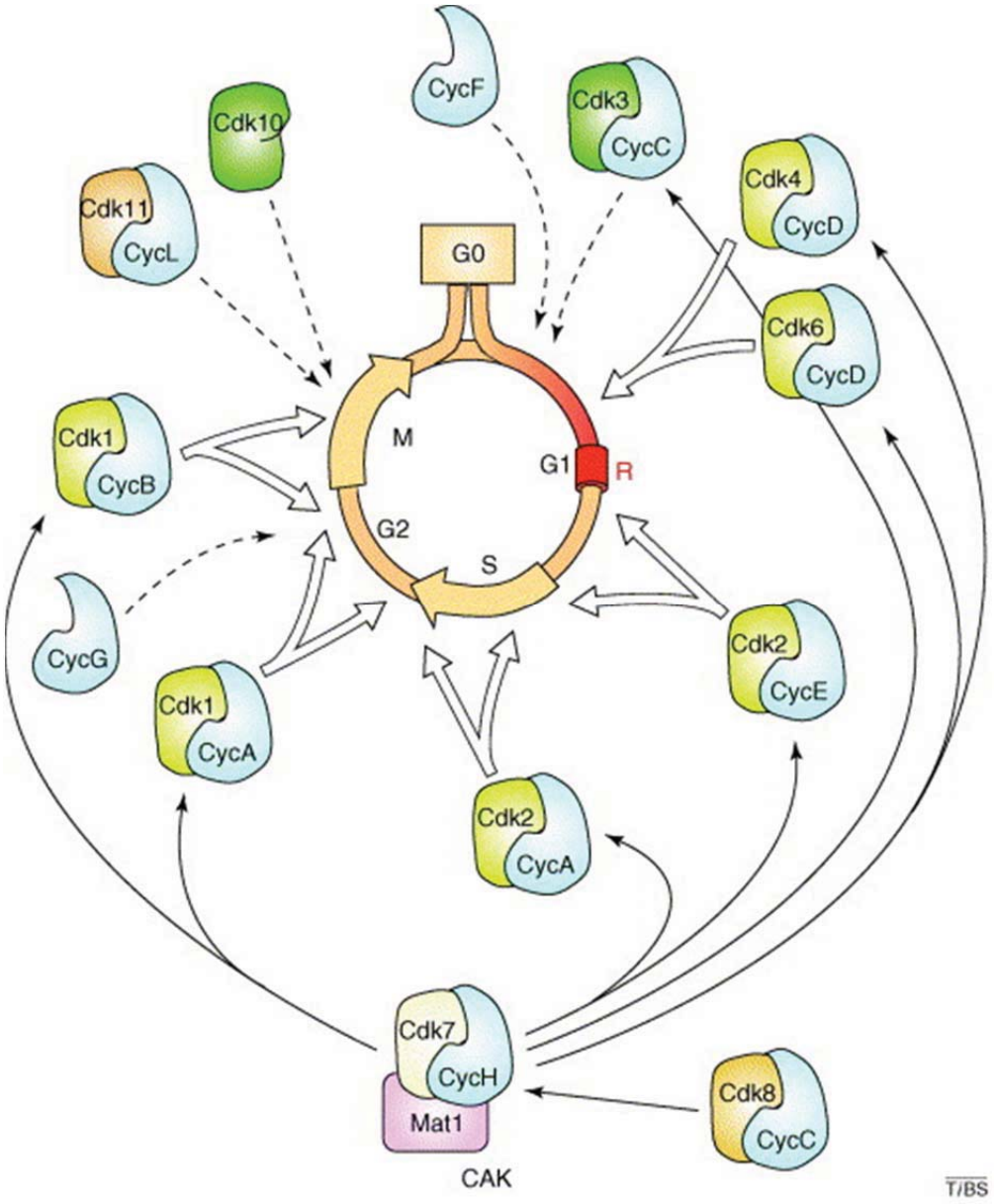


Figure 1. Les quatres phases du cycle cellulaire.

La figure montre les phases du cycle cellulaire ainsi que les complexes actifs des cyclines/CDK. La lettre R indique « le point de restriction ». D'après (44)

"asynchronisées". Environ 30-40% de ces cellules sont généralement en phase S. La proportion de cellules en phase M est appelée l'index mitotique (41).

L'intervalle de temps entre deux cycles cellulaires consécutifs est appelé interphase. Les cellules filles issues d'une division cellulaire peuvent soit quitter le cycle cellulaire et rester dans la phase G0 soit entamer un nouveau cycle de division cellulaire. Les cellules quittent G0 et entrent dans le cycle cellulaire quand elles reçoivent des stimuli mitogèniques, tel que, des facteurs de croissance. À un certain moment dans G1, les cellules entament la division cellulaire. Ce moment est appelé "Point de Restriction ou point R" (45, 46). La base biologique de ce point est maintenant connu (voir ci-dessous). Après le passage par le point R, les cellules subissent la division cellulaire, même en l'absence des stimuli mitogènes.

Les cyclines et les protéines kinases cycline-dépendantes (CDK) représentent deux classes de molécules importantes pour la progression du cycle cellulaire à travers ses différentes phases.

1.2.2 Les Cyclines

Les cyclines se caractérisent par leur production et leur dégradation cycliques et coordonnées avec les différentes phases du cycle cellulaire, d'où l'origine de leur nom. Les différentes cyclines sont identifiées par ordre alphabétique; par exemple cycline A-I, cycline T, etc. Les cyclines A, B, D et E sont les plus importantes dans un cycle cellulaire. Chaque cycline est caractérisée par la présence d'un segment de 100 acides aminés appelé «la boîte cycline» *(cyclin box)*, par lequel les cyclines se lient avec les CDK spécifiques et les activent. Les cyclines contiennent également un domaine dit « PEST », composé de quatre acides aminés (une proline, un acide glutamique, une sérine et une thréonine). Une fois phosphorylé, ce domaine sera reconnu par la ligase E3 de l'ubiquitine et entrainera la dégradation de la cycline. Voir la Figure 2 pour la structure d'une cycline et pour la dégradation des différentes cyclines lors du cycle cellulaire. Le génome humain contient au moins 29 gènes qui pourraient coder pour des protéines contenants les «boîtes cycline» (44). Les cyclines représentent des sous-unités régulatrices des holoenzymes des CDK. La liaison d'une cycline avec CDK libère le site actif de la kinase de l'effet inhibiteur de la la "boucle T" ou "T loop" de la kinase.

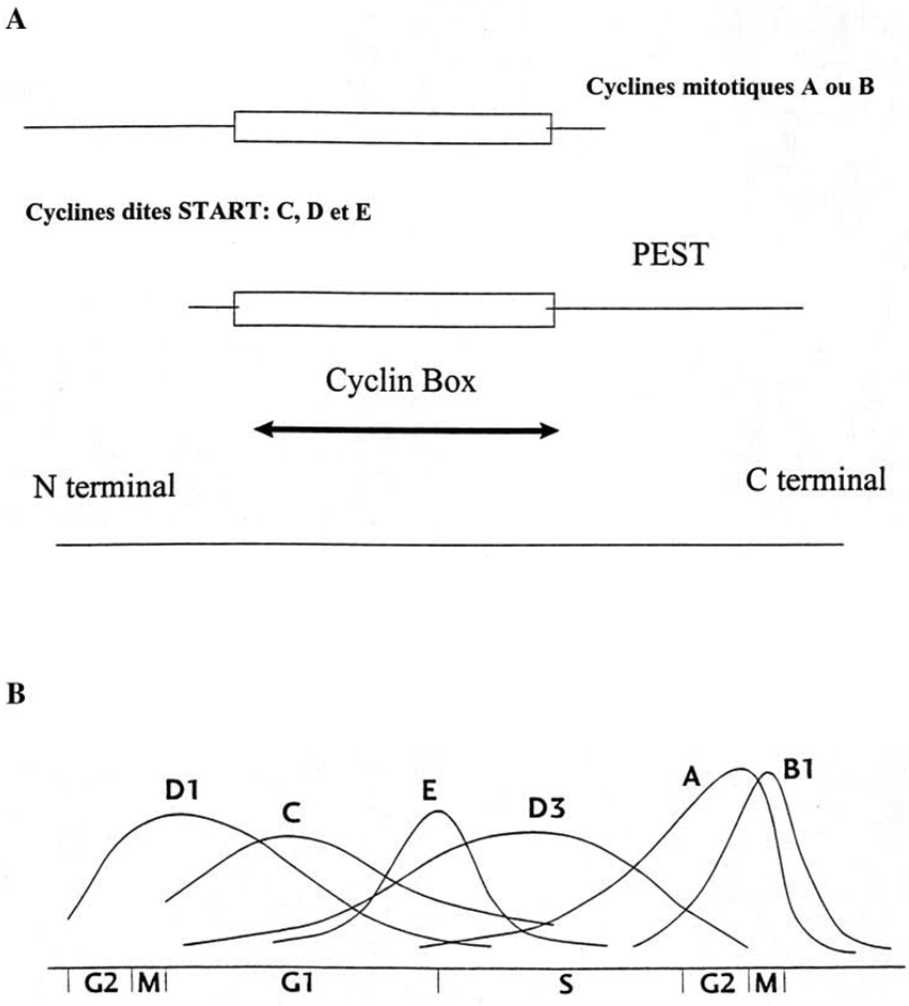


Figure 2 : A. Structure d'une cycline montrant « la boite cycline » ainsi que son domaine « PEST ». B. La Figure montre l'expression et la dégradation des différentes cyclines lors de la progression du cycle cellulaire.

D'après la référence (47).

Les cyclines activent non seulement les CDK, mais définissent également la spécificité de leur substrat. Les cyclines D sont également appelées cyclines «mitogèniques», car elles sont produites dans des cellules de façon transitoire au début de la phase G1 en réponse à des stimuli mitogèniques. Ces cyclines s'associent avec CDK4 et CDK6 et les activent. Les complexes actifs cyclines D/CDK4 et cycline D/CDK6 sont nécessaires pour la transition G0/G1 et le passage à travers le point R. Les cyclines E par contre, sont produites à la fin de la phase G1. Elles se lient aux CDK2 et sont importantes pour la transition des cellules en prolifération de la phase G1 vers la phase S. Ces complexes cyclines/CDK phosphorylent également et inactivent la protéine du rétinoblastome pRb de manière irréversible, permettant ainsi aux cellules d'achever le cycle cellulaire, même en absence de stimuli mitogéniques et ceci correspond au point «R» dans la progression du cycle cellulaire (48). Il est à noter que les cellules dépourvues de pRb (ou qui ont une pRb mutée) ainsi que ses protéines apparentées (p130, p107) ne possèdent donc pas de point de restriction fonctionnelle et elles deviennent immortalisées (49). Le niveau de la cycline E atteint un pic lors de la transition G1/S, et diminue progressivement durant la phase S. Les deux cyclines D et E sont nécessaires pour compléter la phase G1 et entrer en phase S et sont aussi appelées les cyclines de G1. Le complexe cycline A/CDK2 est actif pendant toute la phase S. La cycline A s'associe également avec CDK1 (appelée aussi cdc2) et le complexe demeure actif durant la phase G2. La cycline B est produite vers la fin de G2 où elle s'associe avec CDK1. Le complexe cycline B/CDK1 est important pour la transition G2/M. Ce complexe phosphoryle plusieurs protéines telles que les lamines nucléaires. Une fois phosphorylées, ces lamines vont subir la dégradation. Tous ces événements sont nécessaires à la dissolution de la structure nucléaire et à la progression de la mitose. La cycline B se dégrade subitement avant l'entrée dans la phase M. Les cyclines A et B sont appelées cyclines mitotiques et sont nécessaires pour compléter les phases S/G2/M du cycle cellulaire. Certaines cyclines existent sous différentes isoformes, par exemple, les cyclines A1, A2, B1, B2, D1-3, etc. Leur expression dans les tissus pourrait se faire de manière spécifique. Comme il est le cas pour la cycline A1, dont l'expression est limitée aux cellules germinales (50). Les différentes isoformes d'une même cycline modifient subtilement les spécificités du substrat de leur partenaire CDK. La protéine pRb et ses protéines apparentées sont nommées «protéines de poche» pour «*pocket proteins*», car elles détiennent et inactivent la famille des facteurs de transcription E2F en recrutant des répresseurs de transcription comme des histone-désacétylases (HDAC), qui modifient les queues des histones et des enzymes de remodelage de la chromatine dépendantes de l'ATP, telle que hSWI/SNF (*SWItch/Sucrose Non Fermenting*). Leur phosphorylation par le complexe cycline E/CDK2 libère les facteurs E2F de leurs effets inhibiteurs. Les E2F libérés s'hétérodimérisent avec DP-1 (ou DP-2) (*Differentiation-regulating transcription factor-1/2*) et induisent les gènes nécessaires à la progression du cycle de G1 vers S comme la cycline E, DHFR (dihydrofolate réductase) pour synthétiser les purines, cdc6 et l'ADN polymérase-α. Le relâchement d'E2F est requis pour

produire les matières premières (dNTP, enzymes, etc) et pour synthétiser l'ADN. Parmi les E2F, plusieurs activent la transcription comme E2F1, -2 et -3 ; alors que d'autres qui l'inhibent comme E2F4 et 5 sont exclus du noyau (51). D'autre part, le complexe cycline A/Cdk2 phosphoryle E2F, empêche leur capacité à lier l'ADN et active la transcription. Durant la phase M, il y a peu d'activation de la transcription. Les complexes cycline B/Cdk2 et cycline B/CDK1 phosphorylent les ARN polymerases, le facteur de sélectivité du promoteur SL1, plusieurs composantes de la machinerie transcriptionnelle de base, comme : UBF, TFIIIB (Facteur de Transcription IIB).
Jusqu'à date, de nombreuses CDK partenaires des cyclines sont encore méconnues. Tel est le cas des cyclines qui ressemblent structurellement aux cyclines A et B. La cycline F complémente cdc4, une composante de l'APC (*Anaphase Promoting Complex*). Cette protéine pourrait jouer un rôle dans la sortie des cellules de la quiescence et dans l'entrée dans le cycle cellulaire (52). Les cyclines de G1 et G2 atteignent des pics dans les phases G1 et S, respectivement. Elles constituent des cibles de p53 et semblent avoir un rôle dans l'arrêt en G2/M en réponse aux dommages de l'ADN (53). Ces cyclines se lient à MDM2 (*mouse double minute protein)* et augmentent la dégradation de p53 (54). La kinase activée par la cycline G (GAK), est une sérine/thréonine kinase associée aux cyclines de type G qui est impliquée dans la signalisation à partir du récepteur de facteur de croissance épidermique (55). Les cyclines I, M et S, ne jouent aucun rôle en rapport avec la progression du cycle cellulaire.

1.2.3 Les Kinases Cyclines-Dépendantes (CDK)

Il s'agit d'une classe de sérine/thréonine kinases, dont les activités dépendent de leur association avec différentes cyclines (56, 57). Les CDK sont produites dans les cellules de manière constitutivement et leur niveau demeure plus ou moins constant tout au long du cycle cellulaire, contrairement aux cyclines qui sont produites et dégradées cycliquement à des moments précis.

Les activités fonctionnelles des CDK sont cependant régulées par leurs associations avec les cyclines. Pour une activation et interaction adéquate avec les cyclines, les CDK subissent plusieurs phases de phosphorylations/déphosphorylations au niveau des résidus sérine et thréonine. Chez les eucaryotes unicellulaires, il n'y a qu'une seule CDK dont les activités sont réglementées par les cyclines spécifiques des différentes phases du cycle cellulaire. Toutefois, onze différentes CDK avec leurs unités régulatrices ont été décrits chez les humains et les souris (58). Les génomes de mammifères contiennent également de nombreux gènes qui pourraient coder pour des protéines homologues des CDK appelées "*CDK-like"* sauf que leurs unités régulatrices n'ont toutefois pas été identifiées. Les CDK-1, 2, 4, et 6 régulent la progression du cycle cellulaire. CDK7 s'associe avec la cycline H et

Mat1, et agit comme une kinase activatrice des CDK (CAK; Voire Table 1 pour la liste des CDK ayant un rôle dans la progression de cycle cellulaire) (59). MAt-1 est une sous-unité du doigt RING, qui stabilise l'interaction entre CAK et cycline H. Les CAK phosphorylent et activent d'autres CDK sur des résidus thréonine/sérine conservés. CAK est aussi un composant du facteur de transcription général TFIIH (facteur de transcription IIH) (59). Le complexe cycline C/CDK8 phosphoryle la cycline H et inhibe le complexe cycline H/CDK7. Pour devenir fonctionnellement active, une CDK doit être phosphorylée par la CAK (cyclin H/CDK7) et complexée avec une cycline (60). CDK4 et CDK6 sont activent à la phase G1, tandis que CDK1 et CDK2 le sont dans les phases S et G2 du cycle cellulaire. Certaines CDK peuvent également jouer un rôle dans la prévention de la mort cellulaire. Par exemple, cycline B/cdc2 phosphoryle la caspase 9 sur ser 125 et inhibe l'apoptose (61). La déplétion des CDK6 prévient la phosphorylation de pRb et par conséquent les cellules n'entrent plus en phase S et meurent. L'expression des protéines virales E7 délivre les cellules de leur besoin en CDK6 (62). L'activité du complexe cycline E/CDK2 est également nécessaire pour charger les protéines MCM (*Mini-chromosome maintenance*) sur les origines de réplication et initier la réplication d'ADN (63). Cependant, après initiation de la réplication d'ADN à la phase S, Les complexes cycline E/CDK2 doivent être inactivés pour empêcher une ré-initiation de la réplication. Ceci est accompli par une ubiquitine ligase SCF-Fbxw7 qui va permettre une dégradation protéasomale rapide de la cycline E (voir ci-dessous). CDK2 phosphoryle et inhibe également son inhibiteur $p27^{kip-1}$ (voir ci-dessous). Elle phosphoryle également plusieurs protéines impliquées dans la réparation de l'ADN, la modification des histones, la duplication et la maturation du centrosome. Lors de la dégradation de la cycline E, CDK2 interagit avec la cycline A2 nouvellement synthétisée (ou A1 pour les cellules germinales). Le complexe cycline A/CDK2 phosphoryle plusieurs substrats nécessaires à la progression du cycle cellulaire par la phase S.

À la fin de S, la cycline A s'associe avec CDK1 (voir figure 1). Au cours de G2, les cyclines A sont dégradées et les cyclines B s'accumulent. CDK1 se lie préférentiellement

CDK	Partenaires	Fonctions
Cdk1 (cdc2 ou cdc28 chez les levures)	Cycline A1, A2 Cycline B1, B or B3 Cycline F	Cycline A/CDK1 actif en S et Cycline B/CDK1 actif à la transition G2/M et en M actif à la méiose et à la transition G0/G1
Cdk2	Cycline E Cycline A	Cycline E/CDK2 actif en G1 et à la transition G1/S Cycline A/CDK2 actif en S et à la transition S/G2
Cdk3	Cycline C	Actif en G0/G1 et en G1 précoce
Cdk4 et Cdk6	Cycline D1, D2 or D3	Cycline D/CDK4 et 6 actifs à la fin de G1/S et à la transition G1/S; Nécessaires pour le passage par le point R
Cdk5	Cycline D et G	Sénescence dans les cellules neuronales
Cdk7	Cycline H et Mat1	Les complexes activent CDK1, CDK2, CDK3, CDK4, et CDK6
Cdk8	Cycline C	Cycline C/CDK8 phosphoryle cycline H et inhibe cycline H/CDK7
Cdk9	Cycline T1, T2 Cycline K	Cycline K/CDK9 et cycline T/CDK9 forment P-TEFb (pour l'initiation et l'élongation des transcrits)
Cdk10	?	Progression G2/M
Cdk11	Cycline L1, L2	Progression G2/M

Table 1. Les différentes CDKs, leurs cyclines associées et fonctions en cycle cellulaire.

D'après la référence (44)

avec les cyclines B1 et B2. La cycline B3 semble jouer un rôle que dans la méiose. Le complexe cycline B/CDK1 phosphoryle plus de 70 substrats, y compris la kinase S6, les histones, cdc25, cdc20, Cdh1, la survivine, les lamines nucléaires, les protéines de liaison aux microtubules, HGM-1 (*High mobility group*)-1. Ces événements permettent la dissolution des membranes nucléaires et la fragmentation de l'appareil de Golgi. La Cycline B est dégradée par l'APC (*Anaphase Promoting Complex*) ou par le cyclosome juste avant la sortie de la mitose. CDK1 peut aussi former des complexes trimériques avec les cyclines B et F (64). Cette interaction cycline/cycline induit la localisation nucléaire du complexe. Dans le cerveau, CDK5 s'associe avec p35 et p39 qui l'activent. CDK9 se lie avec la cycline T ou K et forme P-TEFb (*positive-acting transcription elongation factor b*), qui est impliqué dans l'élongation des transcrits par l'ARN pol II (65). Cycline A1 est limitée aux cellules germinales.

CDK10 semble jouer un rôle dans la progression G2/M. Cycline L/CDK11 joue un rôle dans l'épissage et le clivage de l'ARN. En dépit de leur rôle dans la progression du cycle cellulaire, CDK4 et CDK6 sont individuellement indispensables pour assurer la viabilité des souris. Leur abrogation ne concerne que la prolifération de certains types de cellules chez les souris KO. Les souris KO pour CDK4 sont de plus petite taille et développent le diabète (66). De même, les souris KO pour CDK2 survivent, mais en étant stériles. La délétion de CDK1 ou de l'un de ses partenaires cycline A2 ou B1 entraine la mortalité embryonnaire. Il n'est pas fréquent que des cancers se produisent avec des CDK mutants. Cependant, des mutations de CDKI ou des cyclines D et E sont plus courants dans les cancers humains. La plupart des efforts de générations de médicaments ont été dirigés contre CDK2 malgré le fait que les souris KO pour cette kinase survivent, mais tout en étant stériles. L'enzyme semble être importante pour la méiose, mais pas pour la mitose (67, 68).

En plus des cyclines et des CAK, les inhibiteurs (CDKI) des CDK régulent aussi les activités fonctionnelles des CDK. Ces inhibiteurs sont répartis en deux groupes : INK4 et Cip/Kip (*Kinase inhibitor proteins*).

1.2.3.1 Les Inhibiteurs INK4

Leur nom dérive des inhibiteurs de CDK4. Le groupe compte quatre membres: $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, $p19^{Ink4d}$. Ils s'associent tous avec les CDK4 et CDK6 monomériques et inhibent leurs activités kinase en empêchant leur association avec les cyclines de type D; revue dans; (69, 70). Ces CDKI s'accumulent et persistent durant la mi-M-à-G1 jusqu'à ce qu'elles soient phosphorylées par les kinases CDK. Les inhibiteurs INK4 phosphorylés subissent une dégradation ubiquitine-dépendante à la transition G1-S. Grâce à leur capacité

d'inhiber la progression du cycle cellulaire, ces inhibiteurs agissent comme des suppresseurs de tumeurs. Des mutations dans différents gènes INK4 ont été décrites dans de nombreux cancers humains. En outre, INK4a a été impliqué dans l'induction de la sénescence dans les cellules humaines. Un variant d'épissage de ce locus pINK4^{ARF} se lie à MDM2 et l'empêche de dégrader p53 ((71); voir ci-dessous pour plus de détails).

1.2.3.2 Les Inhibiteurs CIP/KIP

Les protéines inhibitrices des CDK/protéines inhibitrices des kinases (Cip/Kip) agissent comme des inhibiteurs des CDK et causent l'arrêt du cycle cellulaire en phase G1. Ce groupe comprend trois membres : p21$^{Cip/Waf-1/Sid-1}$, p27^{Kip1} and p57^{Kip2}. Contrairement aux membres de la famille des INK4, les inhibiteurs CIP/KIP se lient à des complexes cycline/CDK (69, 72). Les structures de ces trois membres sont tout à fait différentes, sauf pour un domaine N-terminal conservé qui permet la liaison avec les CDK et les cyclines. Ceci suggère un rôle distinct pour chacun de ces inhibiteurs. Le gène p21 est induit par p53 en réponse aux dommages d'ADN et il est responsable de l'arrêt du cycle cellulaire dans les deux phases G1 et G2. Quand à p27, elle est habituellement exprimée dans les cellules privées de sérum et de facteurs de croissance. Les niveaux de cet inhibiteur baissent fortement une fois les cellules reçoivent les facteurs de croissance et entrent dans le cycle cellulaire (73). L'expression de p57 est limitée à certains types de tissus et cellules. Le CDKI joue un rôle important dans le développement embryonnaire. Les deux premiers membres du groupe, p21 et p27, inhibent vigoureusement les CDK associés aux cyclines D, E et A. Ils peuvent également inhiber légèrement les CDK1 associés à cycline B.

Il est intéressant de savoir que les CDKI jouent également un rôle dans l'assemblage des complexes CDK4 et 6 avec la cycline D. Toutefois, les complexes cycline/CDK pourraient se former sans ces CDKI (74, 75); revue dans (76). L'activité en aval de cycline E/CDK2 est augmentée par la séquestration de CDKI dans ces complexes. Cette hypothèse est soutenue par le fait que les activités de cycline E/CDK2 sont réduites chez les souris cycline D KO puisque les complexes cycline D/CDK4, et cycline D/CDK6 ne séquestrent plus CDKI (77). La Phosphorylation et les interactions protéine-protéine modifient la structure tridimensionnelle, la localisation cellulaire et donc le potentiel des CDKI d'inhiber les CDK. La phosphorylation de p21 sur son site Thr 57 par CDK2 ou encore par le glycogène synthase kinase 3-beta (GSK)-3β augmente son affinité pour cycline B1/CDK1 à la transition G2/M (78). La protéine nucléaire TAF-1 *(Template activating factor)* se lie à p21, diminue sa capacité à inhiber cycline E/CDK2 et augmente celle pour cycline B/CDK1 (79). Une autre protéine TSG (*Tumor susceptibility gene 101*)-101 se lie également à p21 et augmente sa faculté d'inhibition des CDK (80).

P27 peut être phosphorylée par Src, Abl ou Lyn sur Tyr 74, 88 ou 89 d'une manière dépende du cycle cellulaire. Ces phosphorylations augmentent son affinité pour CDK4, tandis qu'elles diminuent celle pour CDK2. Le CDKI phosphorylé sur Tyr 88 inhibe très faiblement CDK4. Cela pourrait expliquer pourquoi CDKI inhibe CDK4 dans les cellules quiescentes très fortement, mais pas dans les celles en prolifération (81). Ces exemples illustrent parfaitement le fait que les activités inhibitrices des CDK de la famille CIP/KIP, sont modulées au cours du cycle cellulaire par les événements de phosphorylation et des interactions protéine-protéine.

P21 et p57 peuvent inhiber également la synthèse d'ADN en interagissant avec PCNA, l'antigène nucléaire de prolifération cellulaire, qui agit comme étant un facteur de processivité de l'ADN ploymerase delta. Cependant, la phosphorylation de p21 sur certains résidus Ser par PKB (ou Akt), et PKC prévient son interaction avec cet élément de la machinerie réplicative (82). Il a été montré que p27 interagit avec le minichromosome de maintenance (MCM)-7, une composante de la fourche de réplication de l'hélicase, et qu'elle prévient la synthèse de l'ADN, revue dans (70). Outre le changement de leur affinité pour les complexes cycline/CDK et pour d'autres protéines, la phosphorylation des protéines Cip/Kip sur différents résidus modifie également la stabilité des protéines et la localisation subcellulaire. La phosphorylation de p21 sur Thr 145 (par Akt) et Ser 153 (par PKC) favorise sa rétention dans le cytoplasme (82). P27 pourrait également être phosphorylée sur trois sites différents et être retenue dans le cytoplasme.

En raison de leur effet inhibiteur sur les complexes CDK, les CDKI pourraient agir comme des suppresseurs de tumeurs. Ainsi, une faible expression de p27 dans les cancers humains signifie plus d'agressivité. Les études faites chez les souris KO suggèrent que les CDKI pourraient également contribuer à l'oncogenèse, ceci est indépendant de leurs interactions avec les CDK et les cyclines; revue dans (70). Ce mécanisme demeure inconnu et pourrait impliquer un dérèglement de la prolifération des cellules souches. De même, p21 pourrait être oncogénique dans certaines circonstances, par exemple, dans le cas des souris ATM et p53 nulles ainsi que chez les souris normales irradiées, la perte de p21 retarde le développement des tumeurs dû à la diminution de l'apoptose dans les cellules tumorales (83) en l'absence de ces CDKI. Les effets oncogéniques des CDKI peuvent s'expliquer au moins en partie par leur capacité de protéger les cellules contre l'apoptose par divers mécanismes. P21, p27 et p57 peuvent inhiber l'apoptose induite par les voies de signalisation activées par le stress. P21 peut également inhiber l'activation de la caspase-3 (84). En plus de supprimer la transcription médiée par E2F, p21 peut se lier directement à c-Myc et à STAT-3 et empêcher ainsi leurs activités transcriptionelles. Cependant, cet inhibiteur peut également lever la répression du blocage de la transcription par p300/CBP (*CREB binding protein)*.

1.2.3.3 Les Événements de Phosphorylation/Déphosphorylation

Toutes les CDK impliquées dans le contrôle du cycle cellulaire eucaryotique devraient être phosphorylées sur une thréonine conservée (exemple: Thr-160 dans le cas de CDK2, Thr-161 pour CDK1) ou sur un résidu sérine au niveau de la T-loop pour atteindre l'activité enzymatique maximale. L'enzyme responsable de cette phosphorylation d'activation est appelée CAK (*CDK-activating kinase*). En outre, deux kinases Wee-1et Myt-1 inhibent les activités kinase des complexes cycline/CDK en phosphorylant les résidus de Thr et Tyr adjacentes (Thr14 et Tyr 15 dans le cas de CDK1) sur les sous-unités CDK (Figure 3). Une phosphatase à double spécificité cdc 25 (A, B et C) déphosphoryle ces résidus et active les complexes cycline/CDK. La phosphorylation de CDK1 sur la tyrosine 15 coïncide avec le temps de dégradation de la cycline B et la vitesse de sortie mitotique dans les cycles à clivage précoce des embryons de *Xenopus* (85, 86).

La phosphatase Cdc25 retire les phosphates inhibiteurs du complexe cycline B/CDK1 et l'active. Les kinases Wee1 et Myt1 phosphorylent et activent ce complexe. Le dommage d'ADN inhibe aussi le complexe cycline B/CDK1 par plusieurs voies montrées dans ce schéma. D'âpres la référence (87).

CDK1 devient inactive à la fin de G2 suite à sa phosphorylation par les kinases Wee-1 et Mik-1sur Tyr 15. Elle doit être déphosphorylée par la phosphatase cdc25C sur Thr14 et Tyr15 avant qu'elle ne devienne active. Cela déclenche l'entrée en mitose. Le Wee-1 entretient le temps mitotique en protégeant le noyau des cdc2 activées et il coordonne la réplication avec la mitose. La surexpression des activateurs cdc25C ou cdc2 peut dérégler cette coordination. Cdc25C est un composant cytoplasmique qui subit une translocation au noyau à la phase G2/M. Quand il est complexé avec 14-3-3 sigma, cdc25C est exportée à l'extérieur du noyau.

La discussion ultérieure explique très bien que deux types de modifications post-traductionnelles jouent un rôle important dans la progression du cycle cellulaire : les phosphorylations/déphosphorylations sur des résidus tyrosines, sérine et thréonine spécifiques. Les phosphorylations/déphosphorylations activent certaines enzymes (comme les kinases) et elles sont nécessaires pour les interactions entre protéine-protéine (tel est le cas pour les interactions des différentes cyclines et les kinases cycline-dépendantes). Ces phosphorylations peuvent également être nécessaires pour la polyubiquitination et la dégradation de diverses protéines cellulaires reliées au cycle dont les fonctions ne sont plus requises (revue dans (88); les détails de l'ubiquitination et de la dégradation protéosomale suivent dans les sections suivantes). La Figure 4 montre toutes les molécules mentionnées jusqu'à présent et qui jouent un rôle dans la progression du cycle cellulaire.

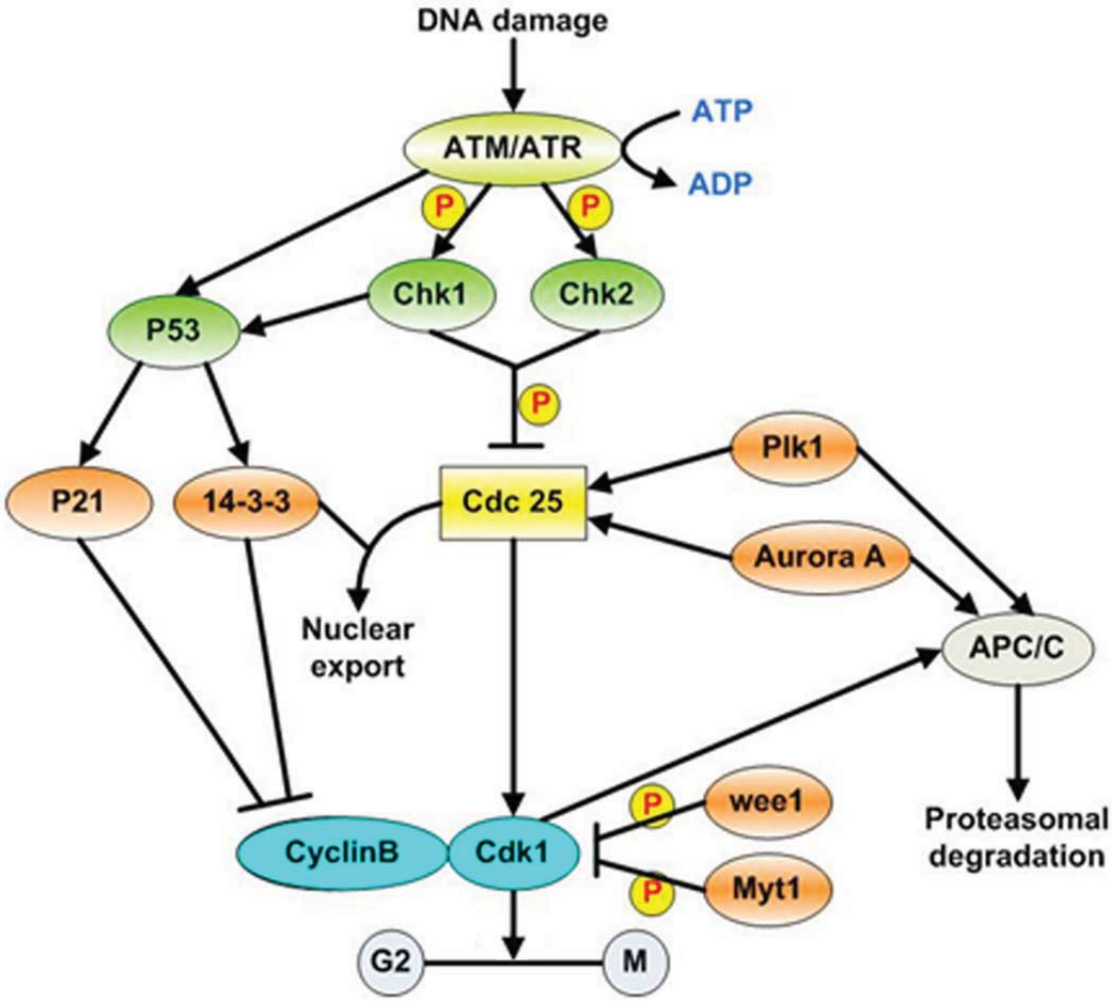


Figure 3. Régulation de l'activation du complexe cycline B/CDK1

La phosphatase Cdc25 retire les phosphates inhibiteurs du complexe cycline B/CDK1 et l'active. Les kinases Wee1 et Myt1 phosphorylent et activent ce complexe. Le dommage d'ADN inhibe aussi le complexe cycline B/CDK1 par plusieurs voies montrées dans ce schéma. D'âpres la référence (87).

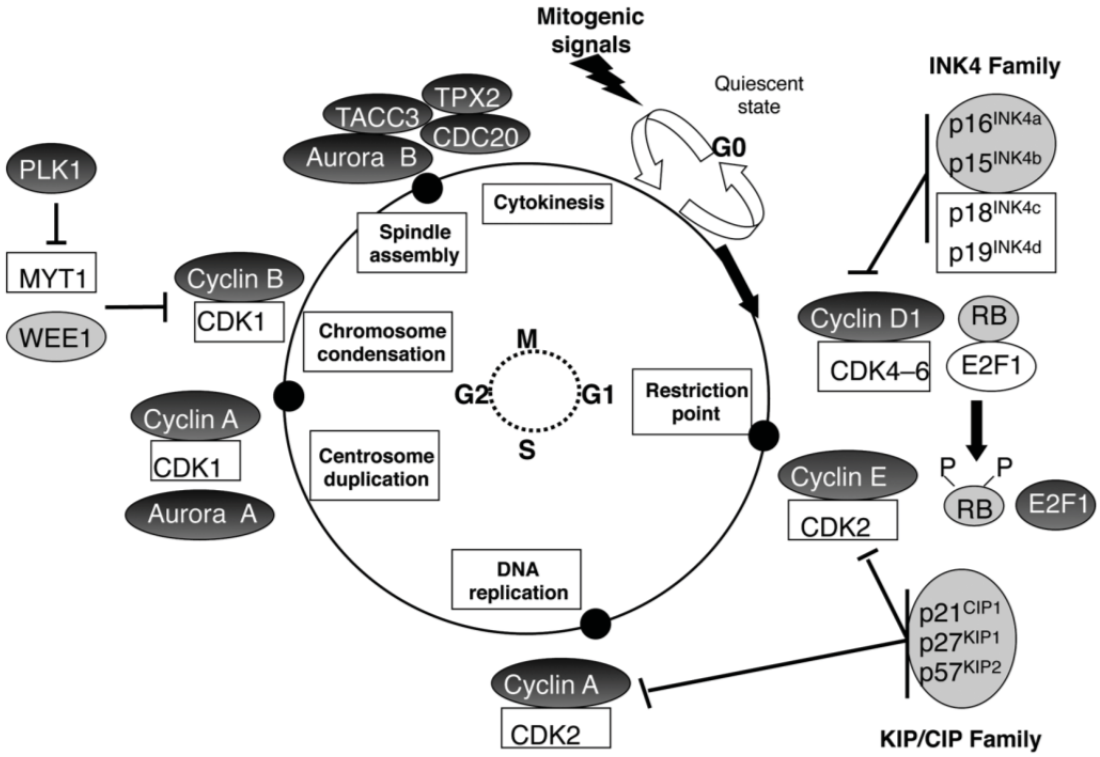


Figure 4. Les molécules jouant un rôle dans la progression du cycle cellulaire.

La Figure montre les cyclines, les kinases, les phosphatases et les inhibiteurs des kinases qui jouent un rôle dans la progression du cycle cellulaire dans les cellules humaines. Les cercles noirs indiquent les molécules qui pourraient se sur-exprimer dans les cellules cancéreuses. Par contre, les cercles en gris indiquent les molécules qui peuvent manquer dans ces cellules. D'après la référence (89).

1.2.3.4 Le Cycle Cellulaire et la Réplication d'ADN.

Durant chaque cycle cellulaire, l'ADN cellulaire se réplique une fois. La réplication de l'ADN commence à partir de l'origine de réplication (ori). Chaque chromosome humain possède plusieurs oris dans les régions dépourvues de nucléosome, contrairement aux procaryotes qui n'ont qu'un seul ori pour chaque chromosome. La machinerie réplicative humaine est assemblée sur la chromatine à chaque ori à la fin de G1. Toutefois, la duplication d'ADN qui ne se produit qu'une fois par cycle cellulaire ne touche que quelque oris. ORC (*Origin recognising complex*) est un complexe hétérohexamèrique des ORC1-6 qui reste attaché à l'ori tout le long du cycle cellulaire. Au début de G1, les niveaux de cdc6 commencent à augmenter. Cdc6 se lie à ORC suivie par le Cdt1 (*CDC10 dependent transcript 1*, un facteur de réplication de l'ADN). Les MCM sont des formes complexes de protéines sous la forme d'un anneau autour de chaque brin d'ADN à proximité de cdc6 (revue dans (90); voir Figure **5**).

Le complexe MCM se compose de six unités MCM (MCM2-7), qui fournissent l'hélicase et des activités primases et pour dérouler la matrice d'ADN. L'assemblage des cdc6, Cdt-1 et MCM à l'Ori est appelée complexe de pré-réplication (pré-C) qui se forme en G1. Après l'assemblage du pré-C, Ori est prêt à «feu» ou à initier la synthèse d'ADN.

L'expression de la cycline E est indispensable pour charger cdc6 et MCM sur ori avant la réplication de l'ADN (91). Cdc6 est une composante hautement régulée du pré-RC. Son activité ATPase est importante pour la réplication d'ADN et son expression est régulée durant les phases S et M par différents mécanismes. L'expression ectopique de cdc6 en G2 retarde l'entrée à la phase M. L'expression de cdc6 relie également la réplication d'ADN avec la mitose**.** Elle prévient l'entrée prématurée dans M si la réplication d'ADN n'est pas terminée. Ceci se fait à l'aide d'un checkpoint qui implique la phosphorylation de Chk1 (92). Les fourches de réplication bloquées par l'aphidicoline nécessitent aussi Cdc6 pour l'activation de CHK1. Cdc6 assure également la sortie de la phase M à temps en inhibant le complexe cycline B/CDK1. Si cdc6 est déplétée en G1, les cellules subissent l'anaphase sans réplication d'ADN. Des ré-initiation aberrantes de la réplication d'ADN se produisent dans les cellules cdc6 mutantes (93). L'activation des complexes cycline E/CDK2 et cycline A/Cdk2 induit l'assemblage de l'ADN polymérase ainsi que d'autres facteurs et initie la synthèse d'ADN. Plusieurs mécanismes sont mis en place pour empêcher la ré-initiation de la synthèse d'ADN en phase S. Les pré-RC sont expulsés au cours de cette phase. La Geminine s'exprime et pénètre dans le noyau au début de S et immédiatement après l'initiation de la synthèse d'ADN. Elle se lie à Ctd1 et la dégrade pour prévenir la ré-initiation de la synthèse d'ADN (94). L'APC dégrade la Geminin au cours de G1. Après l'initiation de la synthèse d'ADN, cdc6 se phosphoryle et se dégrade par SCF (voir ci-dessous).

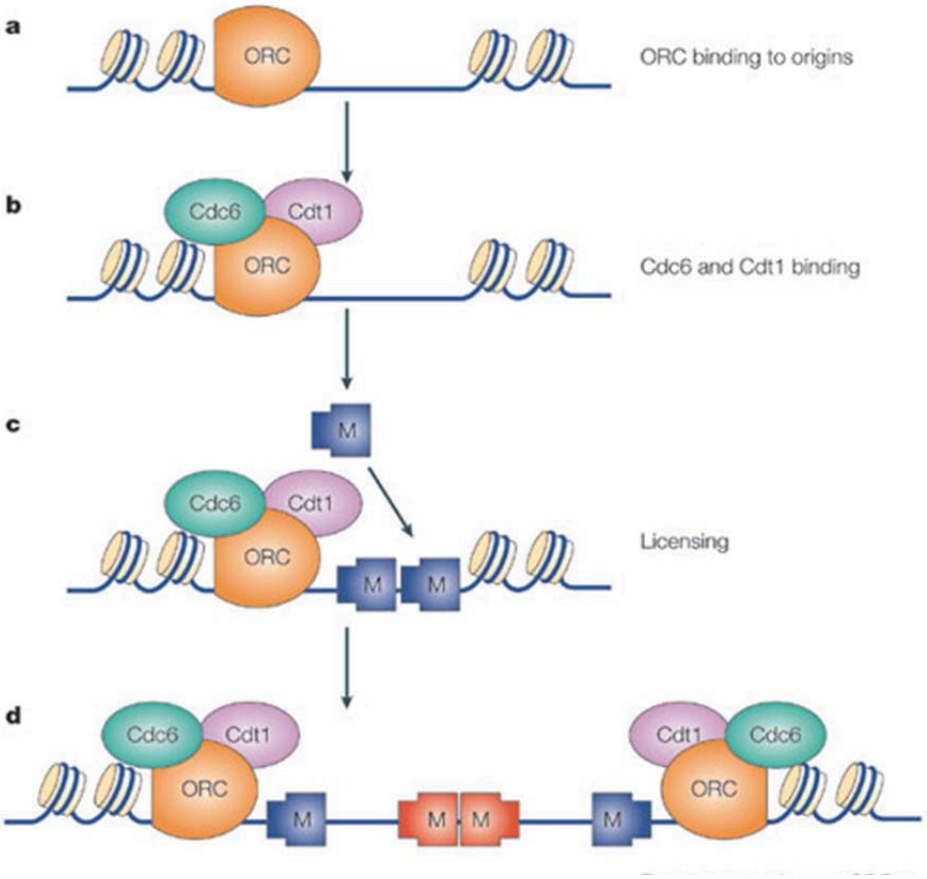


Figure 5: L'assemblage des ORC sur les chromosomes.

Recrutement du complexe de reconnaissance de l'origine (ORC) à l'origine de réplication.

a) L'ORC recrute Cdc6 et Cdt1
b) L'ORC, Cdc6 et Cdt1 agissent ensembles pour charger de multiples protéines hexamères (MCM) 2-7 sur l'origine permettant la réplication de l'ADN.
c) Initiation des complexes-compétents qui se sont probablement formés par back-to-back de deux complexes Mcm2-7.

D'après la référence (95).

La duplication du centrosome, la condensation de la chromatine, la formation du fuseau mitotique et la dissolution de l'enveloppe nucléaire sont des événements qui se déroulent sous le contrôle du temps. Ce sont les complexes cycline A/CDK2 et cycline B/CDK1 qui sont responsables de l'accomplissement de tous ces changements. Le complexe cycline A/CDK2 peut phosphoryler cdc20 et activer l'APC. Alors que cycline A/CDK1cible l'APC entrainant ainsi la dégradation de la sécurine. Emi-1 (*Early mitosis inhibitor-1*) est une autre cible qui interagit avec cdc20 et inhibe APC (96).

1.2.3.5 Le Cycle Cellulaire et la Dégradation médiée par le Protéasome.

Un fonctionnement adéquat du cycle cellulaire repose sur la dégradation de différentes cyclines ainsi que d'autres molécules effectrices à des temps précis. La dégradation de ces molécules se fait par les voies protéasomales dépendantes de l'ubiquitine. Durant ce processus, le substrat qui doit se dégrader est souvent modifié par des phosphorylations au niveau des résidus sérine et thréonine. Pour être dégradé, le substrat phosphorylé est reconnu par le système de dégradation protéasomal médié par l'ubiquitin. Le substrat est ensuite taggé par une chaîne de polyubiquitines conjuguées de manière covalente (sur la lysine48); revu dans (88).

L'ubiquitine est une molécule de 8 kD, produite dans les cellules humaines par quatre différents gènes de façon abondante. Trois enzymes participent successivement au marquage d'un substrat avec l'ubiquitine. La première étape consiste à l'activation de l'ubiquitine par un activateur ou une enzyme E1 suite à une liaison covalente d'une molécule d'ubiquitine à son cystéine par une liaison thioester.

Dans la deuxième étape, E1 transfère l'ubiquitine activée à une enzyme de conjugaison E2. Durant l'étape finale, une ligase ou l'enzyme E3 catalyse le transfert de l'ubiquitine à un résidu lysine sur le substrat.

Le substrat polyubiquitiné est reconnu par le protéasome 26S et il est ensuite dégradé. Avant la dégradation du substrat, une déubiquitinase (DUB) libère des molécules d'ubiquitine pour le recyclage. L'Ubiquitine pourrait être ajoutée à différents résidus lysine sur un substrat (mono-ubiquitiné) en tant que de simples molécules ou encore être ajoutée à une molécule d'ubiquitine déjà fixée sur un substrat (polyubiquitiné). L'ubiquitine pourrait se conjuguée à une autre ubiquitine via une de ses sept résidus lysines (K) (par exemple, K6, K11, K27, K29, K33, K48 ou K63). Cela donne lieu à l'accumulation de chaînes d'ubiquitines avec différentes conformations. Il est connu que les substrats polyubiquitinés sur les lysines 48 et 11 se dégradent par le protéasome 26S. Les protéines monoubiquitinées et multiple-monoubiquitinées ainsi que

les polyubiquitinées par d'autres liens que lysine 11 et 48 ne sont pas reconnues par le protéasome et ne sont pas dégradées. Ces ubiquitinations servent à d'autres fins (tel que la stabilité, la signalisation, ou encore la dégradation par d'autres voies etc; revue dans (97, 98)).

Dans les cellules humaines, il n'y a que deux enzymes E1 (Uba1 et Uba6) mais près de 40 E2 qui ont été identifiées. Cependant un plus grand nombre de ligases E3 ont été découvertes (près d'un millier). Chaque E3 reconnaît certaines cibles spécifiques. Ces ligases E3 peuvent être divisées en deux catégories: Les E3 à domaine RING (*Really Interesting New Gene*) et celles à domaine HECT (*Homologous to E6-associated protein C Terminus*). L'E3 ligases RING catalyse le transfert d'Ub d'une E2 vers le substrat en les regroupant. Ils facilitent simplement le transfert des molécules Ub à partir d'une E2 vers le substrat.

Chaque ligase E3 HECT contient un résidu cystéine conservé sur lequel est transférée la molécule d'Ub qui était sur l'E2. L'enzyme E3 chargée transfert alors Ub sur le substrat. Deux ligases E3 de type RING jouent un rôle important dans la progression du cycle cellulaire. Cela comprend l'APC/C (*Anaphase Promoting Complex /Cyclosome*) et le complexe Skp-1/Cullin/F-box (SCF; voir Figure 6 pour la structure de l'APC/C et du SCF). L'APC/C agit au niveau de la phase G2/ M alors que SCF cible les protéines des phases G1/S/G2.

1.2.3.6 L'APC/Cyclosome.

Il s'agit de multiples sous-unités de l'ubiquitine E3 ligase qui dégradent les cyclines mitotiques juste avant le début de l'anaphase ((99); figure 3). La destruction de ces cyclines est essentielle pour la sortie des cellules de la phase M pendant l'anaphase. Les deux cyclines A et B contiennent un motif qui est la boite de destruction ou D box, et qui est nécessaire à leurs ubiquitinations par l'APC (100, 101). L'APC s'active suite à sa liaison avec cdc20 ou Cdh1 (102). Ces co-activateurs changent la spécificité du complexe pour les substrats ciblés. Parmi les sous-unités d'APC, la phosphorylation des Apc3 et Apc7 augmente leur affinité pour cdc20 et Cdh1. Une autre sous-unité d'APC est l'Apc11, elle agit comme une sous-unité catalytique de type RING. D'autres sous-unités possèdent des fonctions adaptatrices et d'échafaudages.

Les complexes cycline/CDK phosphorylent l'APC et favorisent sa liaison avec cdc20.

Ces phosphorylations inhibent aussi la liaison d'APC avec Cdh1. En outre, l'inhibiteur de mitose Emi-1 (*Early mitosis inhibitor*)-1, qui se lie à APC/cdc20 et l'inhibe pendant l'interphase est également dégradé pendant la phase M.

Cependant, le complexe APC/cdc20 est tenu à l'état inactif par Mad2, qui est libérée des chromatides non attachées. Mad2 ne prévient pas la liaison entre cdc20 et l'APC par

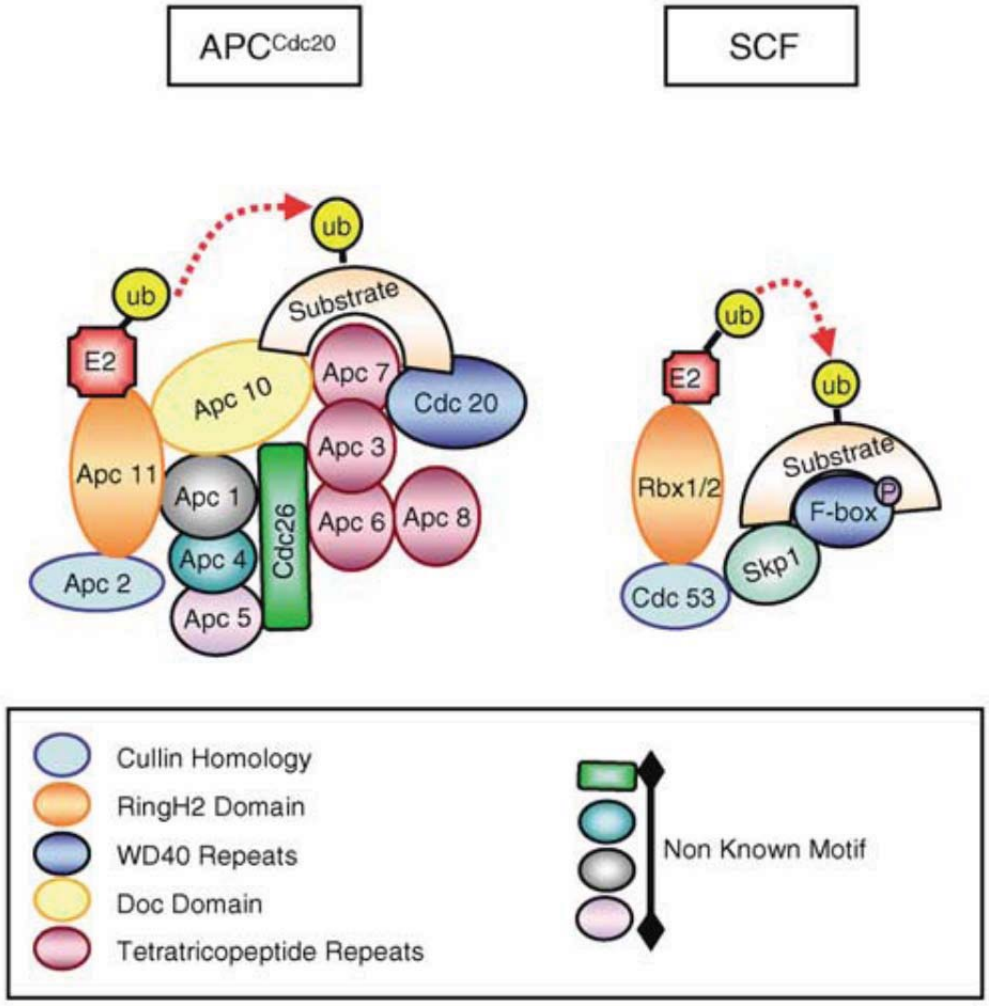


Figure 6. Structure des APC/C et du SCF

Tous deux l'APC/C et le SCF contiennent une Culline, un doigt RING-H2 et une sous-unité WD40. Les protéines ayant des fonctions similaires sont de couleurs identiques. L'APC contient des unités supplémentaires ayant des fonctions inconnues. D'après la référence (103).

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contre, elle prévient son activation. On croit que Mad1, 2, Bub1, BubR1, Mps-1 et BuB3 se lient aux kinétochores non attachés et génèrent le signal d'arrêt. Le complexe du signal qui comprend Mad2 et BubR1 se lie à cdc20 et inhibe l'APC (voire la Figure 7 pour le checkpoint de l'assemblage du fuseau mitotique). BubR1peut également se lier à cdc2 et à Bub3. La nature exacte du signal inhibiteur est encore méconnue. Mad1 recrute Mad2 aux kinétochores. Lorsque toutes les paires des chromatides s'alignent par leurs kinétochores aux microtubules le long du fuseau, ou sur la plaque de la métaphase, le signal d'inhibition n'est plus émis et le complexe APC/cdc20 s'active (102). La perturbation des fonctions de BubR1 dérégule le checkpoint, entraîne une instabilité chromosomique et la malignité. Il a été également montré que BubR1 interagit avec la sécurine et qu'elle la stabilise (104). Il existe une corrélation directe entre l'expression de la sécurine et celle de BubR1 dans les cellules cancéreuses humaines. En se liant à la sécurine, BubR1 peut former un autre point de contrôle mitotique. BubR1 et cdc20 sont toutes les deux phosphorylées dans la phase M. BubR1 possède une activité kinase sérine/thréonine et elle phosphoryle p53 ainsi que cdc20. En outre BubR1 est autophosphorylée aussi bien par Aurora kinase et Polo-like kinase ainsi qu'en réponse aux dommages du fuseau mitotique.

L'APC clive de nombreux substrats autres que les cyclines mitotiques. La sécurine est l'un de ces substrats, elle séquestre la séparase, une protéase thiol et sa dégradation permet de relâcher la séparase. Une fois libre, la séparase va cliver une Sous-unité du Complexe de Cohésion (SCC)-1. SCC1 et trois autres protéines appelées (*Structural Maintenance of Chromosome protein*) Smc-1, Smc-2 et SCC3, forment ensemble une structure en anneau contenant des paires de chromatides nommée cohésine qui retient les paires de chromatides ensemble (105). Le clivage soudain de SCC1 marque l'initiation de l'anaphase, qui s'achève en quelques secondes (Figure 8). Le clivage de SCC1 permet de dégager les chromatides de l'anneau. Les fibres du fuseau se séparent vers leurs pôles respectifs de la cellule. Le complexe APC/cdc20 initie la destruction des cyclines mitotiques à la métaphase tandis que le complexe APC/Cdh1 (Cdh1 est un activateur de l'APC/C) achève cette destruction à l'anaphase.

L'activation d'APC/cdc20 mène à l'apparition de l'anaphase durant laquelle les chromatides sœurs se séparent les unes des autres ainsi qu'à l'apparition du commencement de la cytocinèse. Ceci est accompli par l'inactivation de cdc20 et par l'activation de Cdh1 ((106); revue dans (107)). L'activation d'APC/Cdh1 débute par l'apparition de l'anaphase et se poursuit jusqu'à la phase G1 qui suit la cytocinèse. APC/Cdh1 activé change la spécificité d'APC vers d'autres types de substrats. Il dégrade plusieurs kinases ainsi que d'autres protéines qui sont nécessaires pour la télophase et la cytocinèse.

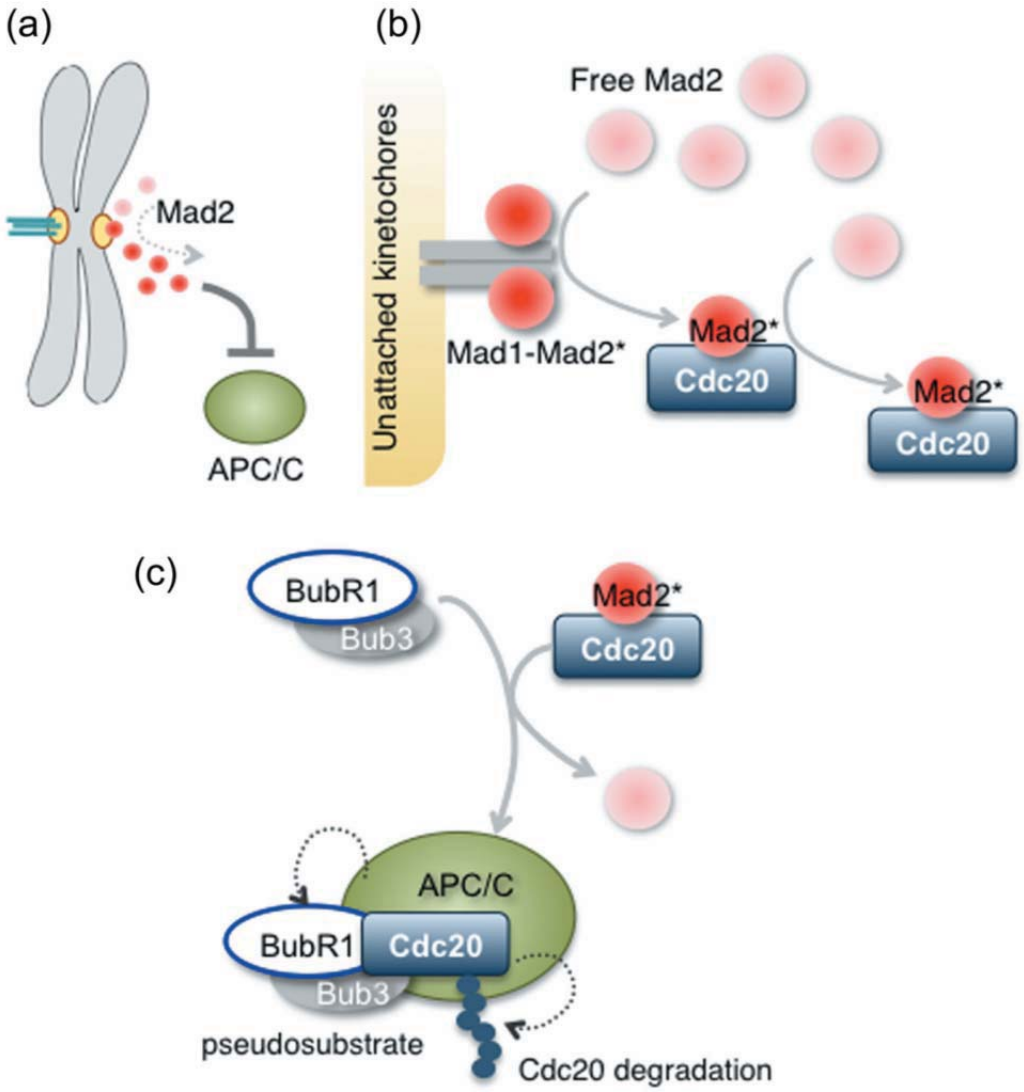


Figure 7 : Le Checkpoint de l'assemblage du fuseau mitotique

La figure montre les composantes du signal inhibiteur envoyé à l'APC des kinétochores non attachées. Mad1, 2, Bub1, BubR1, Mps-1 et BuB3 se lient aux kinétochores non attachés et génèrent le signal d'arrêt. Ce signal comprend Mad 2 modifiée (colorée en rouge foncé). Mad-2 se fixe sur Cdc20 et l'inhibe. Ensuite Mad2 se détache de Cdc20, et BubR1 et Bub3 se fixent sur Cdc20 et le dégradent. L'APC/C ne s'active pas sans Cdc20. D'après la référence (108).

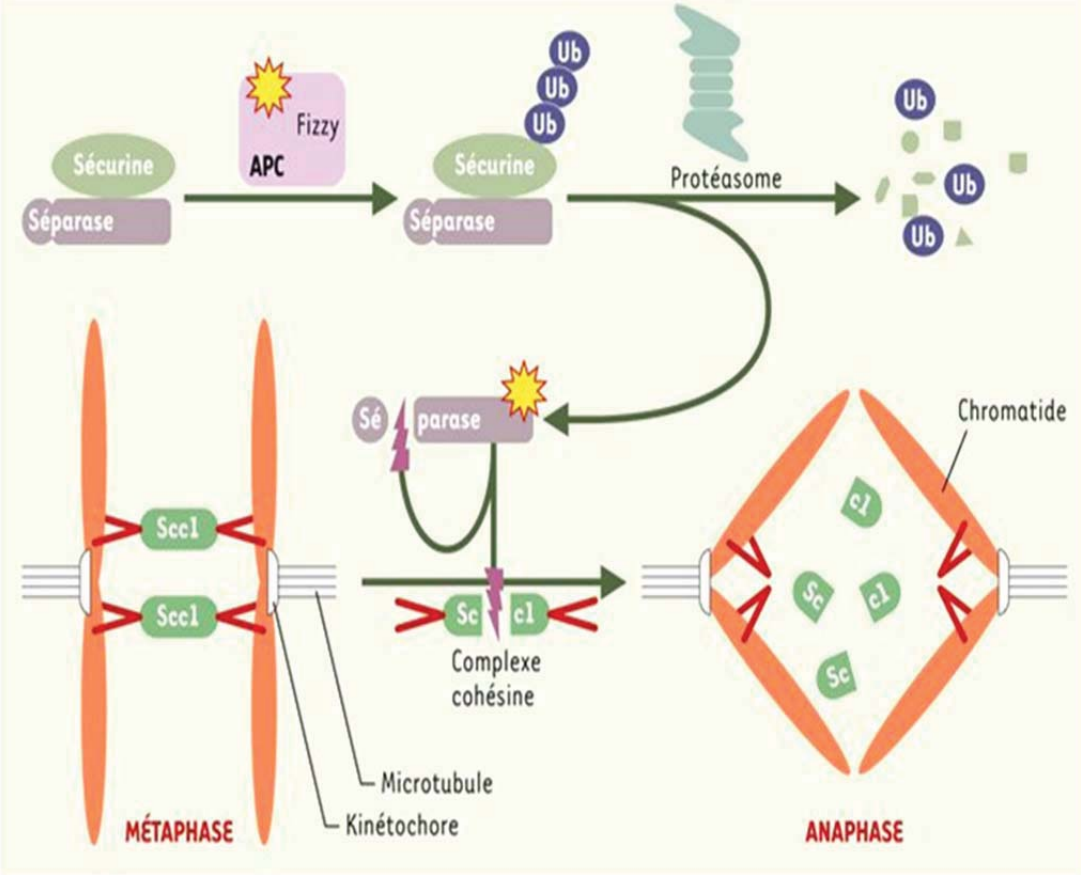


Figure 8 : Le complexe APC règle la transition métaphase/anaphase.

Les deux chromatides sœurs sont attachées par des complexes protéiques appelés cohésines. La sécurine est associée à une protéase de la famille des caspases, la séparase, et la maintient inactive. À la transition métaphase/anaphase, le complexe APC Fizzy (ou Cdh1) actif ubiquitinyle (Ub) la sécurine, qui est alors dégradée par le protéasome 26S. La séparase est alors libérée, s'active et clive la sous-unité Scc1 du complexe cohésine, autorisant la séparation des chromatides sœurs. Le symbole jaune appliqué sur les entités moléculaires reflète un état activé. D'après (109)

Les cellules ré-établissent les conditions pour G1, et peuvent entrer en G0 ou subir une différenciation. Fait intéressant, l'PC/Cdh1 joue un rôle dans l'induction de la différenciation des cellules. Les niveaux de Cdh1 diminuent progressivement dans G1, en raison de l'ubiquitination et de la destruction du soi, au moins en partie (107). APC/Cdh1 induit la polyubiquitination et la destruction de cdc20, Plk-1, Skp-2 et de nombreux inhibiteurs de la différenciation cellulaire. Fait intéressant, la reconnaissance de divers substrats par cdc20 et Cdh1 ne nécessite pas leurs phosphorylations.
Cdc20 reconnaît la destruction des boîtes de motifs (RxxL; D) alors que Cdh1 reconnaît à la fois les boîtes de motifs D et KEN dans les substrats; revue dans (110).
Autres que les cyclines mitotiques, l'anilline est un autre substrat important pour l'APC/C (111). L'anilline est une protéine d'échafaudage et de liaison des filaments d'actine qui est nécessaire à la cytocinèse et qui est localisée dans le sillon de clivage. Les niveaux d'aniline atteignent un pic en mitose mais ils chutent considérablement à la sortie mitotique. L'ubiquitination d'anilline nécessité la boite de destruction et elle est médiée par Cdh1. La kinase, Aurora B est aussi un autre substrat de cette ubiquitine ligase indispensable à la progression mitotique (112). Les niveaux de cette kinase fluctuent au cours du cycle cellulaire. Ils atteignent un maximum lors de la mitose ensuite ils diminuent drastiquement en G1. La kinase Aurora B joue un rôle dans l'assemblage du fuseau mitotique et dans l'attachement des kinétochores aux microtubules opposés lors de la cytocinèse. Une fois la cytocinèse terminée, cette kinase n'a plus d'utilité et elle est ensuite détruite dans G1. La perte de Cdh1 entraine l'accumulation de ses substrats et par conséquent la réplication non programmée d'ADN et l'induction de réponse aux dommages d'ADN. Les cellules déficientes en Cdh1 montrent des signes d'instabilité génomique, des défauts dans la cytocinèse et une polyploïdisation (107).

1.2.3.7 Le SCF.

Le nom du SCF dérive de ses sous-unités qui le composent : Skp-1 (S phase kinase-associated protein-1), Cullin-1 et des protéines de type F-box; revue dans (113). SCF est une ligase E3. Il s'agit de la deuxième ubiquitine ligase E3 qui régule la progression du cycle cellulaire. Elle reste constitutivement active tout au long du cycle cellulaire. Elle dégrade les cyclines et les CDKI dans les phases G1 et S en les reconnaissant selon leur mode de phosphorylation caractéristique. SCF est une ligase E3 prototype du type Cullin-RING Ubiquitine E3 Ligases (CRL; voire la Figure 9 pour la composition des différents CRL). Les CRL sont composées nécessairement de trois sous-unités: Une culline en tant que protéine d'échafaudage, une protéine RING catalytique (également appelée ROC (*Regulator of Cullin*), ainsi qu'une protéine adaptatrice.

Il y a huit sortes de protéines cullines (Cul 1-8) et deux ROC (ROC1 et 2). Toutes les protéines cullines forment individuellement un complexe avec ROC-1. Cependant, culline-5 forme un CRL qui se complexe avec ROC-2. Chaque CRL contient une protéine adaptatrice différente. Les adaptateurs confèrent la spécificité de substrat aux CRL. Par exemple SCF utilise les protéines de la boîte F comme adaptateurs. Il existe près de 70 protéines de la boîte F. Chacune d'entre elles se lie à Skp-1 par l'intermédiaire d'un domaine conservé qui a été découvert pour la première fois dans la cycline F. De même, le SCF^{FBW-7} utilise FBW-7 (*F box and WD repeat domain containing protein-7*) en tant que protéine adaptatrice. L'adaptateur reconnaît et lie certains modèles de phosphorylation à un groupe de protéines spécifiques, et il facilite son ubiquitination par E2 et E3; revue dans (114).

Comme mentionné ci-dessus, SCF^{FBW-7} est actif dans les phases G1, S et G2 du cycle cellulaire. La cycline E constitue l'un de ses principaux substrats. SCF^{FBW-7} reconnaît la cycline E, qui est auto-phosphorylée sur la Ser 384 par le complexe cycline E/CDK dans la phase S et elle est ensuite phosphorylée par GSK-3β sur Thr 380. L'autophosphorylation de la cycline E est requise pour la phosphorylation par GSK-3β. La cycline E duellement phosphorylée est polyubiquitinée par SCF^{FBW-7} (115). Il est intéressant de savoir que le grand antigène T de SV-40 agit comme un inhibiteur pour le substrat de FBW-7. Cela conduit à une augmentation dans l'accumulation de la cycline E et de Myc (un autre substrat de SCF^{FBW-7}) dans les cellules infectées par le virus et favorise la prolifération cellulaire (116). FBW-7 agit comme un suppresseur de tumeur et il est généralement muté dans plusieurs cancers. On compte parmi ses substrats et autre que la cycline E; Myc, Notch, presiniline-1, SREBP (*sterol-response element binding protein*) et Jun. Il est intéressant de noter, que la destruction de Notch et de la preseniline-1 médiées par SCFFBW-7 ne nécessitent pas leur préalable phosphorylation.
Tout comme FBW-7, Skp-2 est une autre composante du SCF qui lui confère une spécificité pour détruire les cibles comme $p27^{Kip-1}$; revue dans (117).
Pour être reconnu par SCF^{Skp-2}, p27 devrait être phosphorylée par la cycline A ou par le complexe cycline E/CDK2 sur Thr 198. (Cks) -1(*Cyclin kinase subunit*) aide Skp-2 dans cette tâche. Comme il est mentionné ci-dessus, le niveau de p27 est plus élevé en G0/G1 et il est plus bas dans la phase S.

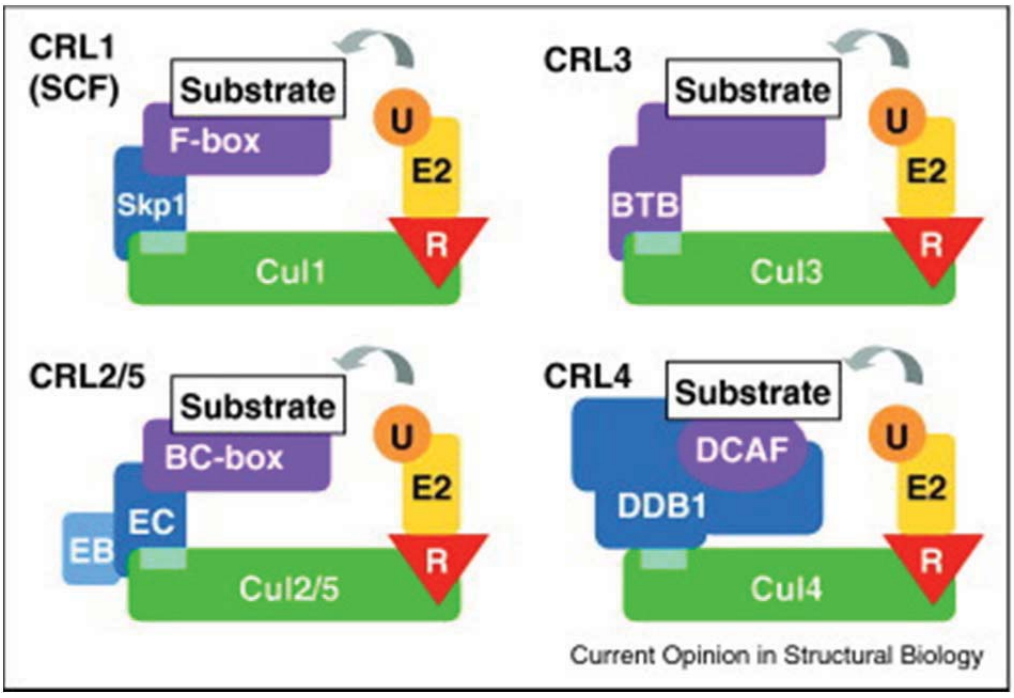


Figure 9. Les composantes des différents Cullin-RING Ubiquitine E3 Ligases (CLR)

La Figure montre les composantes des différents CRL. Le CRL1 ou SCF dégrade différentes molécules en utilisant plusieurs protéines de la boite-F (F-box protéines). Voir le texte pour les détails. R et U indiquent ROC et ubiquitination, respectivement. D'après la (113).

Autrement dit, seuls les niveaux des protéines p27 oscillent au cours du cycle cellulaire, et non pas leurs ARNm. Cela signifie que les niveaux de p27 sont essentiellement sous le contrôle de la dégradation par $SCF^{skp-2-cks1}$. La cycline E, p21, p57 et E2F1 sont aussi d'autres cibles des $SCF^{Skp-2/Cks-1}$. Skp-2 est surexprimée dans de nombreux cancers (117). La β-TRCP (*β-transducin repeat-containing protein*) est une autre protéine F-box qui est importante dans la progression du cycle cellulaire. Elle cible Wee1, Emi-1 et cdc25 pour une destruction et une polyubiquitination médiée par SCF. Fait intéressant, SCF et APC/C interfèrent entre eux de façon considérable lors de la progression du cycle cellulaire. Par exemple, APC/Cdh1 cible Skp-2 pour la dégrader.

1.2.4 La Progression du Cycle Cellulaire.

Lorsque des cellules au repos (G0) reçoivent des facteurs de croissance ou des stimuli mitogéniques, elles synthétisent les cyclines de G1, qui activent CDK4 et CDK6. Ces CDK phosphorylent une protéine suppressive de tumeur, le Rétinoblastome (pRb). Rb ainsi que d'autres protéines associées apparentées suppriment la transcription requise à la progression du cycle cellulaire en se liant et en modulant l'activité des facteurs de transcription E2F, des histone-déacétylases et des complexes de remodelage de la chromatine. La pRb phosphorylée libère le facteur de transcription E2F. pRb contrôle l'entrée en G1/S par la régulation de E2F. Les E2F forment des hétérodimères (E2F1-8) soit avec DP1 soit avec DP2. La pRb appartient à une famille de protéines appelées *'pocket proteins'*. Une fois hypophosphorylées, ces protéines deviennent actives (Figure 10). Elles détiennent et séquestrent E2F dans la «poche» et les conservent dans un état inactif. Comme il est mentionné ci-dessus, la phosphorylation de pRb représente le point R dans la progression du cycle cellulaire. Les E2F activés induisent la transcription des protéines nécessaires aussi bien à l'entrée qu'à la progression de la phase S du cycle cellulaire tel que la cycline E et les enzymes requises à la biosynthèse des nucléotides. Cycline E s'associe avec CDK2 et l'active. Cette activation est essentielle pour la progression vers la transition G1/S. Le complexe cycline E/CDK2 phosphoryle pRB et force les cellules à subir la division cellulaire, même si les signaux mitogènes sont retirés. Généralement, la phase S dure assez longtemps, elle peut prendre jusqu'à 10 heures. Durant cette phase, les cellules vont dupliquer leurs ADN. La cycline A de la phase S est généralement produite à la fin de la phase G1. Le complexe cycline A/Cdk2 est actif au cours de cette phase et il est essentiel à la transition S/G2. Au cours de G2, les cellules vont croitre et dupliquer leurs organelles nécessaires aux cellules filles lors d'une division ultérieure. Vers la fin de G2, la cycline A se lie à CDK1 dans le but de commencer la phase M.

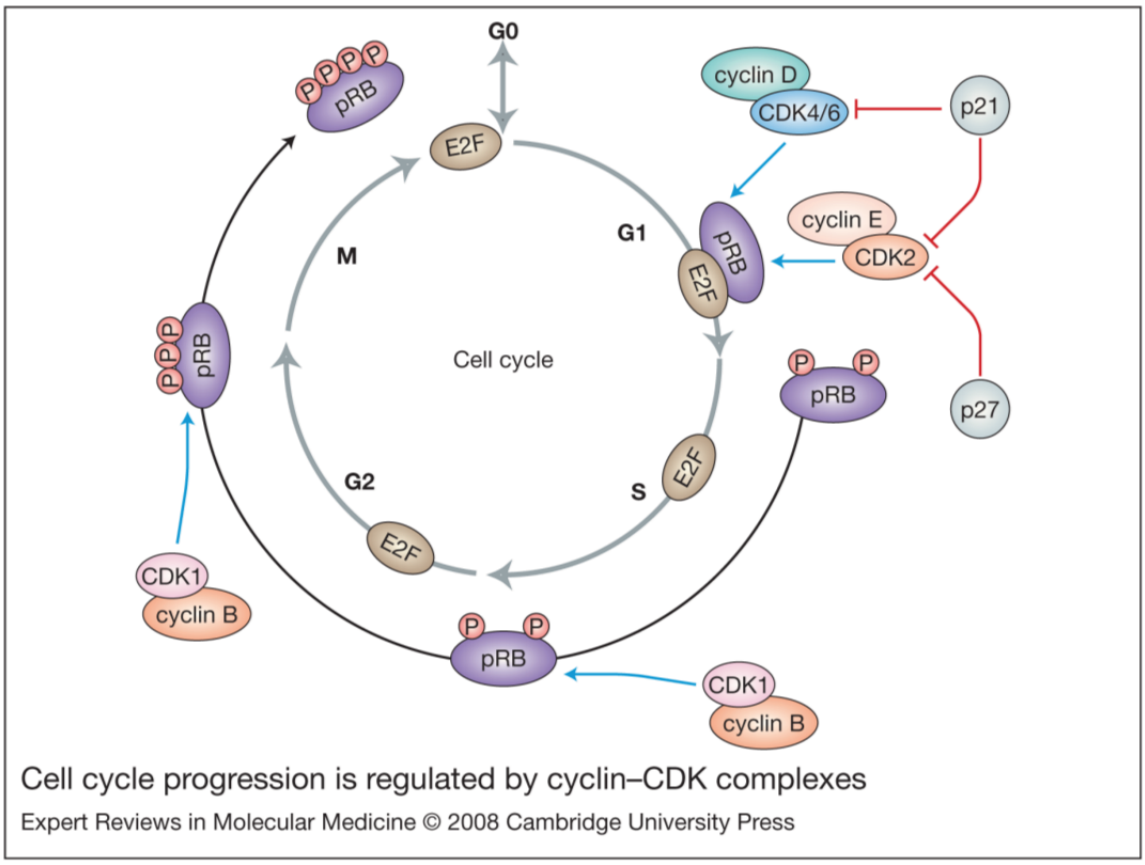


Figure 10: Les membres de la famille des pRb (*pocket proteins*) et la régulation du cycle cellulaire par la voie pRB-E2F.

La pRB se lie et inhibe E2F en G0 et au début de la phase G1. Dans les cellules en prolifération, la phosphorylation de pRB par les complexes cycline/CDK relâche les facteurs E2F, induisant ainsi les gènes qui interviennent dans l'entrée à la phase S. D'après la référence (76).

Après dissociation des membranes nucléaires, les cyclines A sont dégradées et les cyclines B se lient à CDK1. Le complexe cycline B/CDK1 est indispensable pour la progression à travers la phase M. Le début de l'anaphase commence avec la destruction de la cycline B et l'inactivation des complexes cycline B/CDK1.

1.2.5 *Les Checkpoints du Cycle Cellulaire.*

La division cellulaire est une épreuve de grande importance pour l'organisme. Au cours de ce processus, non seulement l'information génétique (l'ADN chromosomique) doit être fidèlement reproduite sans altération sérieuse, mais aussi elle doit être transmise aux cellules filles. Le cas contraire pourrait être catastrophique pour les cellules filles ainsi que pour l'organisme.

Par conséquent, les cellules eucaryotes ont développé plusieurs points de contrôle et des régulateurs de feedback tout au long de la progression du cycle cellulaire. Ces mécanismes de contrôle coordonnent la croissance cellulaire, la réplication d'ADN, la ségrégation des chromosomes ainsi que la division finale de la cellule (cytocinèse). À chaque point de contrôle, la cellule s'assure que chaque étape du processus de la division cellulaire est correctement achevée avant d'entamer l'étape suivante. Si l'un de ces points de contrôle est inactivé, par exemple, suite à des mutations dans les gènes comme p53, les cellules peuvent proliférer en héritant de l'ADN endommagée. Cela peut entraîner une instabilité génomique et le cancer. Les points de contrôle du cycle cellulaire sont décrits ci-dessous :

1.2.5.1 Le Point de Restriction ou R.

Le point de contrôle indique la présence de facteurs de croissance et de stimuli mitogéniques nécessaires pour compléter la division cellulaire.
Cela se produit dans la phase G1. En l'absence de facteurs de croissance, les cellules deviennent quiescentes; c'est la phase G0. Durant cette phase, les cellules cessent de croître mais elles sont métaboliquement actives. Elles réduisent leurs taux de synthèse protéique. Les cellules peuvent demeurer en G0 aussi longtemps, tant qu'elles n'ont pas reçu des facteurs de croissance. Il arrive qu'elles «se mangent elles-mêmes» par autophagie et qu'elles subissent l'apoptose si elles restent affamées pendant de longues périodes de temps. Si les cellules reçoivent des facteurs de croissance et des substances mitogèniques en G0, elles commencent à se multiplier et elles entrent en G1. Si au cours de G1, elles reçoivent assez de stimuli mitogéniques, elles synthétisent la cycline D, activent CDK2 et CDK4, phosphorylent pRb et activent les facteurs E2F, on dit alors

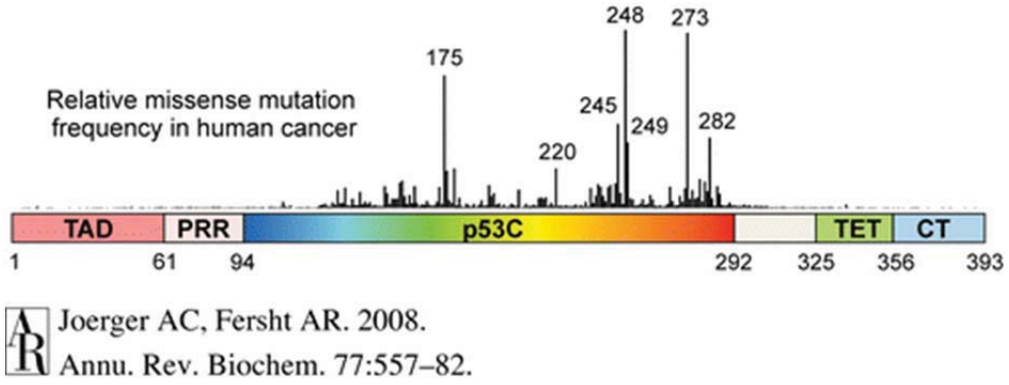
qu'elles ont franchi le point «R» (montré dans le Figure 1). Après avoir traversé le point R, les cellules s'engagent dans un cycle de division cellulaire même si les stimuli mitogèniques sont retirés. Par conséquent, ce point est aussi appelé "point d'engagement".

1.2.5.2 Le Checkpoint du Dommage d'ADN en G1.

Ce point contrôle l'entrée à la phase S. Il arrête la progression du cycle cellulaire suite aux dommages d'ADN et permet aux cellules de réparer les dégâts. L'arrêt du cycle peut être temporaire ou permanent (la sénescence). Si les dommages sont importants et les cellules ne parviennent pas à le réparer, elles pourraient subir l'apoptose. Ce checkpoint est médié par p53, qui fait également partie intégrante d'autres points de contrôle du cycle cellulaire en réponse aux dommages d'ADN.

P53 dispose d'un domaine liant une séquence spécifique d'ADN à la région centrale de la protéine et d'un domaine de forte stimulation de la transcription à son extrémité N-terminale (voire la Figure 11, pour les différents domaines de la protéine). La protéine fonctionne habituellement sous forme de tétramères. P53 se lie par ces sites et agit comme un activateur transcriptionnel. Plusieurs gènes ont des sites de liaison à p53 dans leurs régions promotrices, tels que : Bax, p21Waf-1//Cip-1 et la cycline G. Les produits de ce gène, donnent lieu soit à la mort cellulaire soit à l'arrêt du cycle cellulaire en phase G1. Il est intéressant de savoir que p53 peut également être recrutée pour des complexes de transcription au niveau des promoteurs qui n'ont pas de sites de liaison à p53 et qu'elle est capable de réprimer leurs activités. P53 est également induite en réponse aux dommages d'ADN et suite à une réplication non programmée d'ADN. L'Activation de p53 implique plusieurs étapes: la stabilisation, l'accumulation, les modifications post-traductionnelles comme la phosphorylation, la méthylation et l'acétylation. P53 induit l'expression de GADD-45, qui joue un rôle dans l'excision et la réparation des *mismatchs*· P53 a été même associée à une activité exonucléase 3'-5 'et à l'activité de *proof reading*. Elle peut également provoquer l'apoptose en induisant l'expression de plusieurs protéines pro-apoptotiques, comme; Bax (*Bcl-2-associted X protein*), Fas, PUMA, NOXA, Scotin, caspase-6, etc, ainsi qu'en réprimant la transcription de plusieurs protéines anti-apoptotiques, telle que Bcl2, BclXL**.** On ne sait toujours pas quand ni comment est prise la décision entre l'arrêt ou la mort cellulaire. Des signaux de prolifération provenant des lymphocytes pourraient influencer les résultats de l'activation de p53 en réponse à l'irradiation-γ (118). P53 joue un rôle dans la réparation de l'ADN. Elle s'active en réponse aux dommages d'ADN. Le gène p53 est localisé dans la région 17q13 du chromosome humain. Comme p53 joue un rôle important dans la prolifération cellulaire, il n'est pas surprenant que plus de la moitié des tumeurs en soient mutées. D'autres montrent souvent des mutations au niveau des gènes impliqués dans la voie de p53, par exemple, au niveau d'ARF; revue dans (119). La plupart de ces mutations se produisent dans le domaine qui se fixe à la séquence d'ADN. Le produit du gène mutant est incapable de

se lier à l'ADN et d'induire la transcription. Grace à son rôle important dans la stabilité du génome, p53 a été reconnue comme « gardienne du génome ».
En réponse aux dommages d'ADN, l'hypoxie, la déplétion des nucléotides, le stress métabolique, les infections virales et l'activation oncogénique, p53 induit l'expression des gènes impliqués dans l'arrêt du cycle cellulaire, dans la réparation d'ADN et dans la mort cellulaire. En plus de se lier à des séquences d'ADN spécifiques à p53, le domaine d'activation N terminal se lie également à des facteurs associés à TBP (*TATA box binding protein*), TAFs (*TBP-associated factors*), TAFII31 et TAFII170.



Joerger AC, Fersht AR. 2008.
Annu. Rev. Biochem. 77:557–82.

Figure 11 : Structure de p53 avec ses différents domaines.

TAD : Transcription activation domaine, PRR : Proline-rich region, p53C : Central or DNA-binding domain, TET : Tetramierization domaine, CT : C-terminal Domain. Voir le texte pour plus de détails. D'après la référence (120)

P21 fait partie des gènes cibles de p53. P53 peut aussi réprimer la transcription de nombreux gènes qui possèdent une boîte TATA (TATA box) et qui sont dépourvus des séquences spécifiques de p53, comme les gènes de l'IL-6 et pRb. En outre, p53 réprime la transcription par l'ARN polymérase I, II et III. P53 induit l'arrêt du cycle cellulaire en G1 via p21, en inhibant les activités des CDK. La liaison et l'inhibition de PCNA par p53 sont importantes pour le chargement de l'ADN polymérase δ et ε sur les matrices d'ADN. En inhibant PCNA, p21 inhibe également la réplication d'ADN dépendante de PCNA, toutefois, sans inhiber la fonction réparatrice d'excision des nucléotides de polymérase et PCNA (121, 122).

P53 peut aussi arrêter les cellules en transition G2/M. À la fin de G2, CAK active cycline B/CDK1 pour faire progresser les cellules en phase M. CDK1 est inhibée par p53 dans les cellules endommagées par différents mécanismes: par l'induction de l'expression des trois inhibiteurs de CDK1 qui sont; p21, GADD-45 et 14-3-3σ. GADD-45 se lie à CDK1 et inhibe sa liaison avec la cycline B, alors que 14-3-3σ inhibe l'activation de CDK1 en se liant et en séquestrant cdc25C, l'activateur de CDK1, dans le cytoplasme. En outre, p53 réprime la transcription de CDK1 par le facteur de transcription NY-F et se lie avec la cycline B empêchant ainsi son association avec CDK1. Par conséquent, p53 prévient les catastrophes mitotiques en inactivant CDK1 et en empêchant la phosphorylation de ses substrats. P53 induit également l'expression de l'ADN topoisomérase II α requise pour le remodelage et la décaténation des chromosomes en leurs formes mitotiques. Sans cette topoisomérase, les cellules s'arrêteront en G2 (123); revue dans (124).

Plusieurs kinases (ATM, ATR, DNA-PK) peuvent phosphoryler p53 au niveau de plusieurs résidus comme; Ser15, The18, et Ser37. À leur tour, CHK1 et CHK2, peuvent phosphoryler p53 à son résidu Ser20 augmentant ainsi sa tétramèrisation, sa stabilité ainsi que son activité de transcription (125, 126). L'endommagement d'ADN induit la phosphorylation au niveau de Ser15 et Ser20. Quand p53 est phosphorylée sur sérine 15 et sérine 20, elle ne lie pas MDM-2 (voir ci-dessous) et par conséquent, elle n'est pas dégradée (127). La phosphorylation de p53 par CAK au niveau de Ser392 affecte défavorablement son effet suppresseur de croissance, reflétant ainsi ce qui se produit dans les cellules cancéreuses. Par contre, sa phosphorylation au niveau de Ser46 contrôle sa capacité d'induire l'apoptose (128). P53 est également acétylée par p300 et CBP acétyltransférase (CBP/p300) au niveau de la lysine 382 dans les cellules humaines et elle est déacétylée par SIRT-1 (*Sirtuin-1*) (129). L'acétylation favorise l'accumulation de p53 ainsi que sa liaison à l'ADN, néanmoins elle nécessite sa préalable phosphorylation sur Ser15. MDM2 inhibe l'acétylation de p53 alors que $P19^{ARF}$ (ou simplement ARF) antagonise cet effet inhibiteur; revue dans (130). Des études récentes ont montré que le degré de phosphorylation de p53 par différentes kinases détermine sa capacité à se lier à CBP/p300 (131).

P53 est régulée négativement par MDM2. Dans les conditions physiologiques, p53 se lie à MDM2 ensuite elle est rapidement dégradée. Fait intéressant, p53 induit ce gène comme un

mécanisme de feed-back négatif. MDM2 se lie au domaine de transcription-activation de p53, et agit comme une ligase E3 du domaine RING pour p53. Le suppresseur de tumeur ARF antagonise MDM2. Il se lie à MDM2 et inhibe la dégradation de p53 médiée par MDM-2 (132). L'ARF active ATM et CHK2, en réponse aux dommages d'ADN et aux cassures d'ADN double brin. Les kinases activées phosphorylent MDM-2 entrainant ainsi sa dégradation. L'ARF n'est pas produite dans les cellules qui prolifèrent normalement. Cependant, l'activation des oncogènes et/ou la prolifération cellulaire non programmée induit sa production. MDMX (connu aussi comme MDM-4) est un autre régulateur négatif de p53; revue dans (133). La Figure 12 montre plusieurs molécules régulatrices de p53. L'activation de p53 dépend des taux de ses régulateurs négatifs (MDM-2 et MDM-4). ATM et CHK2 activées phosphorylent également MDMX sur trois résidus sérines conservées et entrainent ainsi sa dégradation.

Cette dégradation est essentielle pour la stabilisation et l'activation de p53. La prévention de la phosphorylation de MDMX, entraine une résistance aux radiations et une susceptibilité à la lymphomagenèse induite par c-Myc chez les souris. MDM-4 se lie également au domaine de transcription-activation de p53. MDM2 et MDM4 effectuent aussi quelques fonctions non-redondantes. La surexpression de ces régulateurs négatifs nuit aux fonctions de p53. MDM4 possède un domaine RING, mais aucune activité ligase E3. MDM2 et MDMX s'hétérodimérisent et accomplissent de meilleurs fonctions qui antagonisent p53. Quand elle est sous forme d'hétérodimère avec MDM-4, MDM-2 dispose d'une meilleure activité ligase E3 (134, 135).

P53 est une protéine multifonctionnelle fascinante. En plus de son rôle de médiateur des points de contrôle du cycle cellulaire et d'inducteur de réponse aux dommages d'ADN, p53 régule la survie cellulaire, la prolifération, la sénescence, la différentiation et la reprogrammation métabolique; revue dans (136, 137). Même les mutantes p53 transcriptionnellement inactives exercent des fonctions par translocation vers la mitochondrie. Il est de plus en plus évident que p53 remplit de nombreuses fonctions importantes pour la survie des cellules (comme, anti-oxydant et anti-autophagique).

Il existe deux autres membres de la famille p53 : p63 et p73. Ces deux gènes agissent comme des facteurs de transcription et ils activent des sous-groupes de gènes qui se chevauchent. Contrairement aux souris p53 KO, les souris p73 KO présentent des défectuosités sévères au niveau des fonctions neurologiques et immunologiques. Les souris p63 KO montrent aussi des anomalies du développement. Tous les membres de la famille p53 produisent différentes isoformes (α-η). Ces isoformes résultent de l'épissage alternatif et de l'utilisation de promoteurs alternatifs. Beaucoup de ces isoformes ont des

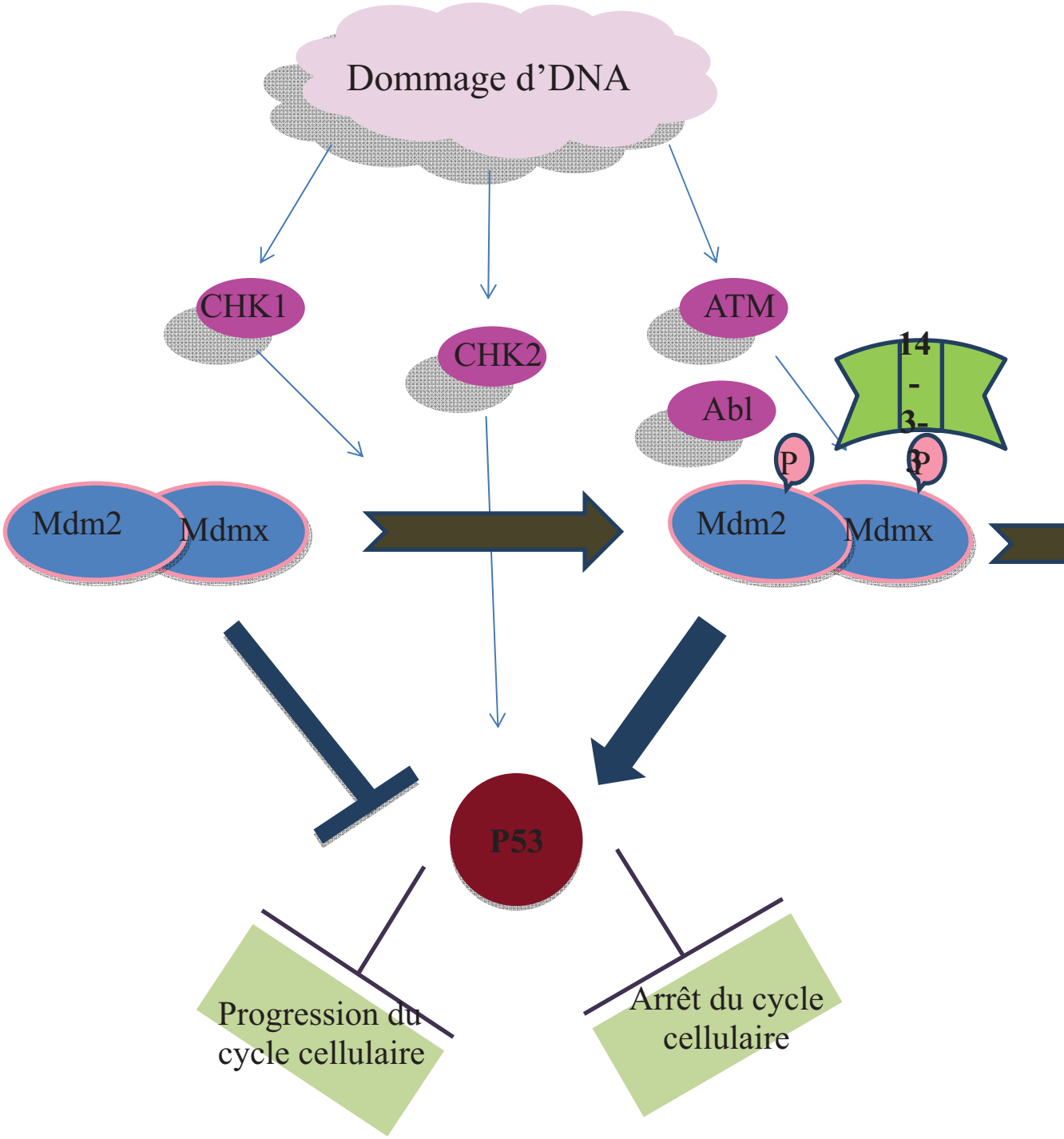


Figure 12: Régulation de p53 par MDMX et MDM2.

Le dommage d'ADN permet la phosphorylation de MDMX qui va interagir avec d'autres protéines et permettre la dégradation de MDMX et de MDM2 ainsi que l'activation p53.

N-terminales tronquées et des activités de transcription manquantes. Ils antagonisent les fonctions des protéines de type sauvage et agissent donc comme des oncogènes. Les facteurs (Ras par exemple) qui favorisent la production de ces isoformes tronquées, favorisent la formation de tumeurs et affectent l'évolution clinique de la maladie; revue dans (138).
La protéine p73 pleine longueur ayant une fonction trans-activatrice intacte est pro-apoptotique induit l'expression de Bax, Fas et PUMA. La protéine virale E1a induit p73, et PUMA dans les cellules p53 mutantes (139, 140). La protéine PUMA est pro-apoptotique avec un seul domaine de BH-3. Elle peut causer la translocation de BAX aux membranes mitochondriales. PUMA pourrait être induite aussi par p53, par contre, E1a l'induit de façon indépendante de p53. Une surexpression de p73 a été observée dans de nombreux cancers. P73 qui est également induite par p53, induit également le stress du RE en induisant l'expression de Scotin, une protéine faisant 23 kD et résidente dans le RE (141).

Des études récentes ont montré que Delta N-P73 joue un rôle dans la neuro-protection. Et ce, en inhibant la phosphorylation de tau (une protéine associée aux microtubules) médiée par JNK et la formation d'enchevêtrements neuro-fibrillaires observée dans les cerveaux de patients atteints par la maladie d'Alzheimer; revue dans (138). L'isoforme se concentre dans le site de dommages d'ADN et inhibe la signalisation de la réponse aux dommages d'ADN par l'intermédiaire de P53BP-1 (142).
Tout comme p73, p63 produit également ses propres N-terminales délétés. Tous deux p63 et p73 sont induites en réponse aux dommages d'ADN et au stress cytosolique. Les gènes cibles de p63 et p53 sont BAX, PUMA, p21, MDM2, la cycline G, GADD45, IGFBP-1, 14-3-3σ, etc.

1.2.5.3 Le Checkpoint du Dommage d'ADN en G2.

L'activation de ce point de contrôle arrête la progression du cycle cellulaire en G2. Il s'active au moindre dommage d'ADN cellulaire (143). L'exposition des cellules aux radiations ionisantes et à la lumière UV par exemple, provoque des cassures au niveau de l'ADN simple brin (SSB) et double-brin (DSB) respectivement. Rad17 agit comme un facteur de chargement spécifique au point de contrôle en réponse aux cassures d'ADN double brin et recrute un complexe protéinique 9-1-1 pour le site endommagé. Le complexe, appelé *checkpoint clamp complex* (CCC), contient Rad1, Rad9 et Hus1. Ceci est rejoint par le complexe Rad3-Rad26. Rad3 active BRCA1, qui à son tour phosphoryle et active CHK1. CHK1 activée phosphoryle directement les phosphatases cdc25C. Cdc25C phosphorylée se lie à la protéine 13-4-4 et se déplace vers le cytoplasme. La séquestration de cdc25C dans le cytoplasme la rend inactive. Une fois activée, CHK1 active à son tour

Myt1 pour le phosphoryler sur Tyr15 et inhiber CDK1. Les dommages d'ADN se traduisent donc par l'inactivation de CDK1 et l'arrêt du cycle cellulaire en G2.

1.2.5.4 Le Checkpoint de l'Assemblage des Chromatides.

Ce point de contrôle, contrôle la sortie de la phase M. Il veille à ce que toutes les chromatides soient attachées et alignées le long du fuseau métaphasique. Les chromatides non attachées relâchent Mad2, qui prévient l'activation de l'APC (Voir la Figure 7). Cette activation est requise pour la dégradation de la sécurine et la séparation des chromatides les unes des autres. L'activation du checkpoint mitotique empêche l'initiation de l'anaphase. Par conséquent, les cellules s'arrêteront alors dans la phase G2/M du cycle cellulaire.

1.2.6 Le Cycle Cellulaire et le Centrosome.

Le Centrosome se compose d'une paire d'organites appelés centrioles qui sont entourées d'une masse granuleuse dans le centre de la cellule près du noyau (144). Des filaments ayant l'aspect de rayons (faits à partir des monomères α et β-tubuline) convergent à partir de chaque centriole. Une forme particulière de la tubuline, appelé γ-tubuline se retrouve dans le centrosome. Cette tubuline à la capacité de nucléer les autres tubulines. Durant la division cellulaire, les centrioles se dupliquent de façon conservative. Un nouveau centriole est formé par division de l'ancien en deux. Une fois matures, les deux centrosomes vont organiser l'assemblage du fuseau mitotique et sa polarité (Figure 13). Les cellules dépourvues de centrioles pourraient s'arrêter en phase G1 suite aux erreurs dans la ségrégation chromosomales. Le centrosome contrôle l'organisation cellulaire ainsi que la polarité. Les centrosomes se dupliquent et se déplacent vers les extrémités opposées du noyau lorsque les cellules se préparent à la mitose et répliquent leur ADN. Lorsque la membrane nucléaire est rompue, les centrosomes agissent en tant que pôles pour le fuseau mitotique: une machine moléculaire très dynamique qui lie les chromosomes et les distribue entre les cellules en division avec une précision stupéfiante. Les microtubules (MT) sont organisées en deux voies antiparallèles avec leurs extrémités négatives (–) sur les pôles et leurs extrémités positives (+) sur l'équateur. Toutefois, les centrioles ne sont pas essentiels pour l'assemblage du fuseau et à la ségrégation des chromosomes, c'est juste qu'ils confèrent plus de précision au processus.

1.2.7 La Cytocinèse.

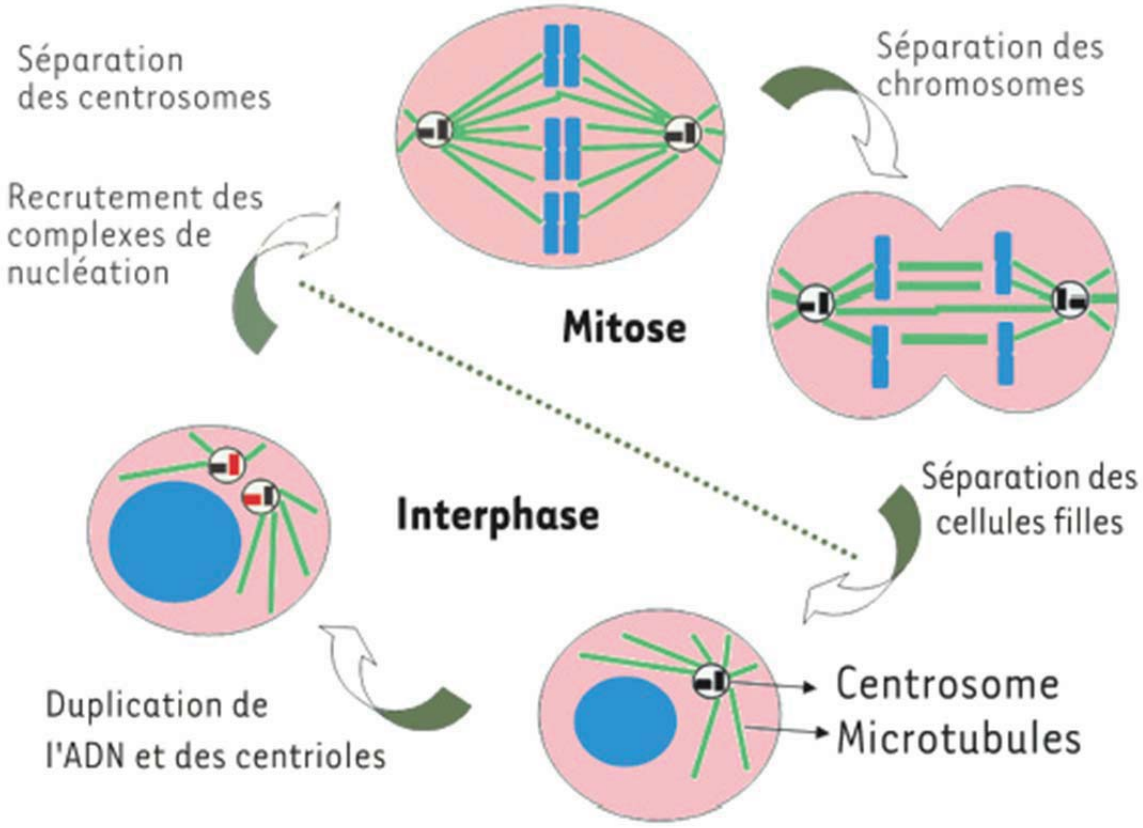


Figure 13: Le centrosome fonctionne comme un centre organisateur des microtubules cellulaires.

Le centrosome est constitué de deux centrioles entourés du matériel péricentriolaire où sont nucléés les microtubules. Le dédoublement du centrosome se fait à l'interphase par duplication des centrioles: chaque nouveau centrosome est composé d'un centriole parent et d'un centriole néoformé (en rouge). D'après (145)

Il s'agit de la phase finale du cycle cellulaire pendant laquelle les deux cellules filles se séparent physiquement l'une de l'autre; revue de (146, 147). La cytocinèse répartit le cytoplasme et son contenu entre les deux cellules filles. Un anneau contractile des filaments d'actine et de myosine, situé perpendiculairement au fuseau mitotique, se développe dans le cortex (juste adjacent à la membrane cellulaire). L'anneau contracte et forme le sillon de clivage, qui pénètre à l'intérieur jusqu'à un petit pont « le corps intermédiaire» et relie les deux cellules filles. La Figure 14 montre une cellule en cours de cytocinèse.

La cytocinèse commence par la formation du fuseau mitotique à l'anaphase. Le fuseau spécifie la localisation à l'équateur, où la F-actine et la myosine-II s'assemblent pour former l'anneau d'actomyosine contractile; revue dans (148-150). La dégradation brutale de cycline B à l'anaphase conduit à la déphosphorylation, la multimérisation et l'activation de la protéine associée au fuseau mitotique, PRC1 (*Protein regulator of cytokinesis*). Cette protéine interagit avec un moteur de type kinésine, KIF4, qui permet de transloquer PRC1 vers les terminaisons plus des microtubules antiparallèles inter-digitées du fuseau mitotique. À ces extrémités, PRC1 regroupe les microtubules pour former les faisceaux de la zone médiane qui agit comme une plate-forme pour localiser d'autres protéines. Les protéines du complexe passager du chromosome CPC (*chromosome passenger-protein complex*) et les membres du centralspindlin (CS) sont deux groupes de protéines importantes qui s'accumulent dans la zone médiane. Les deux complexes sur les microtubules du fuseau central se chevauchent les uns avec les autres. Autre que PRC1, le complexe cycline B/CDK1 phosphoryle également la protéine mitotique (MKLP)-1 (*mitotic kinesin-like protein*) et empêche son attachement avec les microtubules du fuseau (151). ECT-2 (*epithelial cell transforming gene 2*) est une autre protéine, qui est phosphorylée et maintenue inactive. Le complexe CPC comprend la survivine, la borealin et INCENP (*inner centromere protein*). Ce complexe recrute et régule l'activité d'Aurora B, une sérine/thréonine kinase. Il se situe sur les kinétochores lors de la prométaphase, pour migrer vers les microtubules du fuseau de la zone médiane vers la position du sillon formant la membrane plasmique et ensuite le corps intermédiaire durant la transition métaphase/anaphase (152). L'activité kinase d'Aurora B est requise pour lier l'actine. Elle phosphoryle la protéine GAP (*GTPase-activating*) pour Rac, RacGAP, elle inactive Rac et les protéines liées à cdc42, toutefois, elle active RhoA. La déphosphorylation d'Aurora B est également nécessaire pour sa translocation vers les microtubules centraux du fuseau.

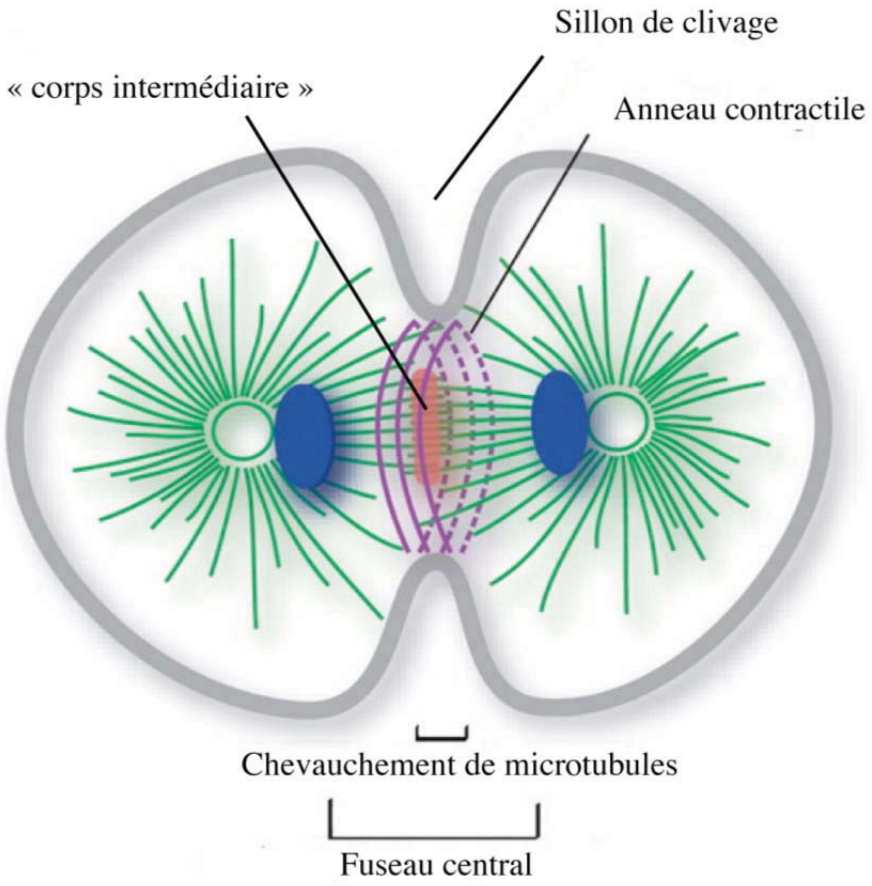


Figure 14 : Représentation d'une cellule en cytocinèse

Les microtubules sont schématisés par les structures en vert alors que l'ADN est en bleu. D'après (147).

Le Centralspindlin (CS) est un complexe hétérotétramérique formé par la protéine mitotique (MKLP)-1 et les protéines Rho activant la GTPase ainsi que le gène de Cytocinèse CYK-4 ou MgcRacGAP (153); revue dans (149). Le complexe régule le groupement des microtubules et recrute également des protéines nécessaires à l'abscission. Plusieurs autres protéines associées avec le fuseau central sont transloquées à la zone médiane. En plus de KIF4, MKLP-2 transloque également ces protéines du fuseau vers la zone intermédiaire. MKLP-2 (Rab-6 kinésine) est une composante du transport vésiculaire. Rho activées recrutent l'assemblage d'actomyosine vers le sillon (PLK)-1 (*Polo-like kinase*), qui est une sérine/thréonine kinase qui phosphoryle MKPL-1 et 2 (149, 154). Aurora B aussi phosphoryle MKLP-1. Ces phosphorylations sont nécessaires pour leur translocation vers le fuseau central. Aurora B phosphoryle également MgcRacGAP et induit l'activité GAP *in vitro*. Elle phosphoryle également la GEF-H1 (MT-binding GEF) et active RhoA. (Voire la Figure 15 pour les interactions moléculaires lors de la cytocinèse).

L'une des protéines qui se transloquent vers le sillon de la zone médiane lors de la cytocinèse est la protéine d'échafaudage, appelée protéine moyenne ou anilline (155). Elle est située à l'intérieur du noyau à l'interphase (figure 16). Elle répond à des signaux spatio-temporels. Lors de la dissolution de la membrane nucléaire, elle se relocalise vers le cortex en métaphase et dans le corps central en télophase (Voir Figure 16). L'anilline devient filamenteuse en anaphase, elle se lie et regroupe les F-actine. Elle interagit également avec les septines (protéines d'échafaudage qui se lient à Rho A) et avec la forme phosphorylée de MRLC (*myosine II regulatory Light Chain*). L'anilline se lie aux septines et les recrutes dans le sillon de clivage. Elle possède un domaine PH (*pleckstrin homology*) à son extrémité C-terminale par lequel elle s'ancre à la membrane. ECT-2 est une GEF (facteur d'échange du nucléotide guanine) qui active Rho A, en fonction de l'activation localisée de RhoA, elle entraine l'assemblage de l'anneau contractile d'actomyosine. ETC-2 contrôle également la localisation d'anilline dans le cortex en métaphase. L'anilline organise différentes composantes de la machinerie cytokinètique comme les septines, les filaments d'actomyosine, Rho A, etc. Elle permet leur ancrage dans la membrane plasmique d'une part et aux extrémités plus des microtubules du fuseau central d'autre part. Plusieurs protéines régulant la dynamique d'actine s'accumulent dans le sillon comme la cofiline, la profiline et les membres de la famille diaphanous.
La signalisation RhoA joue un rôle dans l'assemblage de l'anneau. En interagissant avec des protéines homologues à la formin et à la profiline, RhoA favorise la polymérisation de la G-actine à la F-actine. RhoA active Rho kinase (ROK) et Citron kinase (CITK). Ces deux dernières phosphorylent la chaine légère régulatrice de la myosine (MRLC), et régulent l'assemblage et la contractilité de l'anneau. Le rôle du CIT-K pourrait être spécifique au tissu. Il diphosphoryle MRLC et pourrait jouer un rôle important dans la réticulation des filaments d'actine. Au cours de l'ingression du sillon, l'activité Rac doit être réprimée. Rac est un membre des petites GTPases de la famille Rho. Ceci est réalisé par RacGAP. Rac est

l'antagoniste de Rho, et il inhibe la CIT-K. L'activité de Rac diminue tandis que celle de Rho augmente dans le sillon lors de la cytocinèse; revue dans (146).

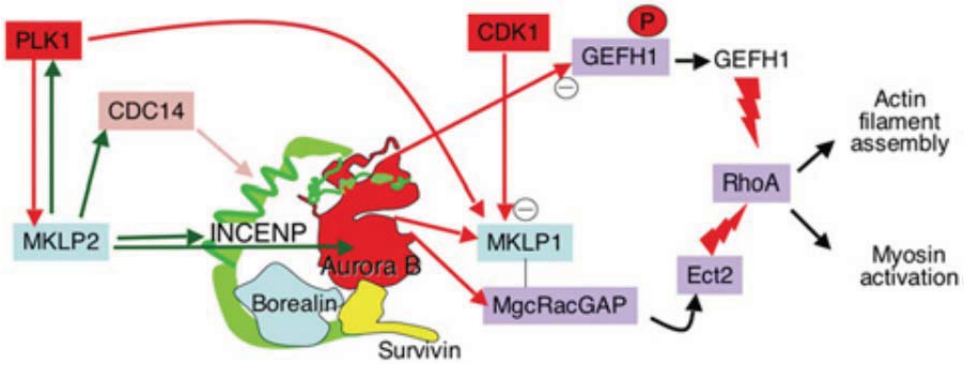


Figure 15. Les interactions moléculaires lors de la cytocinèse

La Figure montre les interactions moléculaires qui se manifestent lors de la cytocinèse et à la fin de la phase M. Les flèches rouges indiquent les phosphorylations et les vertes indiquent les protéines requises pour la localisation du fuseau central. D'après (152).

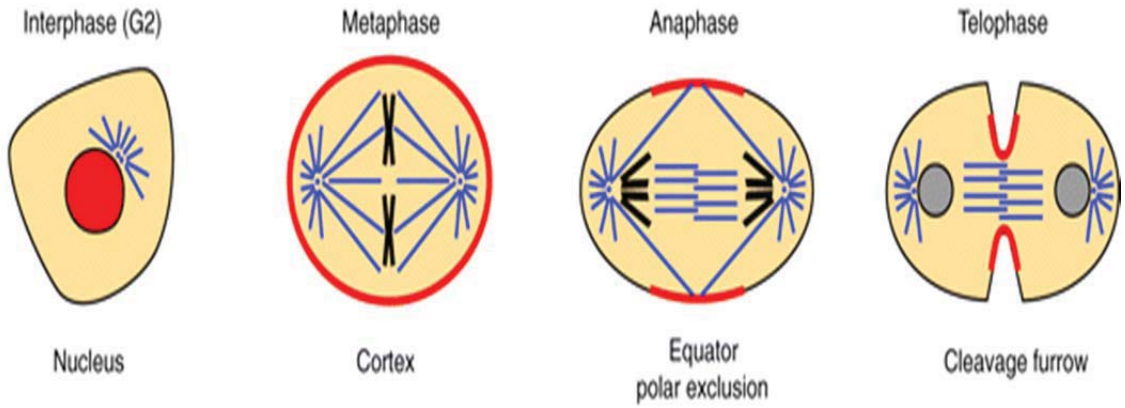


Figure 16: La localisation de la protéine Anilline au cours du cycle cellulaire lors de la cytocinèse

La répartition de l'Anilline est représentée en rouge, les noyaux sont en gris, les chromosomes en noir, et les microtubules, les centrosomes et les pôles du fuseau en bleu. D'après (156).

RhoA, les septines et l'anilline forment des complexes dynamiques qui subissent des cycles d'assemblage et de démontage. La phosphorylation de la chaîne légère de myosine (MLC) sur Ser 19 de la myosine II est le moteur de l'anneau contractile. Le glissement progressif des mini-filaments de myosine le long des filaments d'actine fournit la force de contraction pour l'anneau; revue dans (149). La machinerie de fusion membranaire est requise pour fournir la membrane supplémentaire au sillon rentrant et compléter ainsi la membrane plasmique pour chacune des cellules filles. Les vésicules du trafic membranaire font ce travail. Ce processus nécessite des protéines associées; les syntaxines, les complexes coatomer et les Rab GTPases.

A la fermeture du sillon, F-actine disparaît et forme un anneau stable du corps intermédiaire. L'anneau contient l'anilline et les septines et il se clive par abscission. Lors de l'abscission, la protéine centrosomale CEP55, joue un rôle fondamental (146). Elle interagit avec MKPL1, TSG-101 (un composant de l'ESCRT-1 (*endosomal sorting complex required for transport-1*)), Alix (une protéine associée à ESCRT) et elle les recrute au corps intermédiaire. Ces protéines recrutent en outre l'ESCRT-III. Il a été montré qu'une spastine ATPase joue un rôle dans la rupture des microtubules lors de l'abscission (157). CHMP-1B (*chromatin-modifying protein 1B*), le composant d'ESCRT-III interagit avec la spastine qu'il recrute pour le corps intermédiaire. Les complexes ESCRT jouent un rôle dans la biogenèse des corps multivésiculaires et le tri de leur cargo; revue dans (158). TTC-19 (*Tetratricopeptide repeat domain 19*) est une protéine centrosomale qui se concentre dans le corps intermédiaire par le biais de son interaction avec CHMP-4B (*chromatin-modifying protein 4B*), une composante d'ESCRT-III. Elle réglemente l'oligomérisation du CHMP-4B. Dans le corps intermédiaire, les lipides membranaire de type phosphatidylinositol 3-phosphate sont générés par une PI-3K de classe III. Ces lipides recrutent la protéine d'échafaudage; une protéine centrosomale qui contient un domaine FYVE (Fab-1, YOTB, Vac-1 and EE-1-domaine; FYVE-CENT) pour le corps intermédiaire (159). La translocation de ces protéines d'échafaudage est médiée par KIF-13A, l'un des moteurs kinésine-3. Elle recrute TTC-19 et ESCRT-III à l'intérieur du corps intermédiaire. Là où les protéines ESCRT causent l'abscission membrane entre deux cellules filles.

Les cellules prennent une heure environ pour terminer la cytocinèse depuis le début de l'anaphase. Une cytocinèse incomplète entraine la régression du sillon de clivage. Les cellules issues d'une cytocinèse ratée endurent l'un des quatre sorts suivants: soit elles restent polyploïdes et s'arrêtent à la prochaine phase G1, soit elles subissent l'apoptose, soit elles vont extruder un noyau, ou encore elles attendent la mitose suivante et aboutissent à 4 cellules filles (145). Il est à noter que la multi-nucléation en soi ne provoque pas d'arrêt. Si ces cellules binucléées continuent la division cellulaire sans compléter la cytocinèse (endoréplication), elles peuvent devenir octoploïdes. Le fait d'avoir plus de deux séries de chromosomes (polyploïdie) entraîne souvent la formation de cancers dus à l'instabilité chromosomique. Les cellules peuvent se séparer avec un fuseau mitotique unipolaire ou multipolaire, si elles en ont un ou plus de deux centrosomes, respectivement. Les cellules filles résultantes disposent d'un nombre anormal de chromosomes (aneuploïdie). La perte d'importants gènes suppresseurs de tumeurs chez les cellules aneuploïdes favorise la carcinogenèse; revue dans (160). En outre, les mutations de p53 peuvent aboutir à un nombre anormal de chromosomes.

1.3 P53 dans les Cellules Infectées par les Virus.

Les cellules eucaryotes répondent généralement à des infections virales en activant p53; revue dans (161). P53 activée pourrait causer la mort prématurée des cellules ou encore inhiber la réplication virale. Par conséquent, les virus à ADN codent souvent à des protéines qui lient et inactivent p53. La prolifération inappropriée des cellules peut également induire p53. L'antigène moyen T du virus du polyome active la voie ARF-P53 (162), alors que le petit antigène T l'inactive (163). Les petits virus à ADN causant des tumeurs comprennent HPV, SV40, le virus du polyome, et les adénovirus. Ils sont tous capables de détourner la machine de synthèse d'ADN de la cellule hôte à leur propre profit. La synthèse d'ADN viral externe peut induire une réponse aux dommages d'ADN grâce à la structure habituelle constituée de terminaisons d'ADN double brin nus. La réplication virale peut être accompagnée de cassures d'ADN. Ceci active la voie ATM qui provoque l'induction de p53 et sa phosphorylation en son aval. Les petits virus à ADN causant des tumeurs ne peuvent pas synthétiser tous les gènes nécessaires à la réplication de l'ADN viral. Ils infectent les cellules quiescentes et induisent la réplication non programmée de l'ADN des cellules infectées. Ils répliquent ainsi leur propre ADN en même temps que l'ADN cellulaire. Tous ces virus codent pour des protéines virales capables d'inactiver pRb indépendamment des complexes cycline/CDK. Ces protéines comprennent PyMT (*polyoma virus middle T*), SV40LT (*Simian virus-40 large-T*), HPVE7 (*human papillomavirus E7*) et E1A des adénovirus. L'entrée non programmée des cellules en phase S peut également induire p53. L'ARF induit p53 en réponse au stress oncogénique. E1A et PyMT induisent l'ARF. Malgré qu'il soit un gène cible d'E2F, ARF n'est pas induit dans les cellules en prolifération normale. PyMT active également la voie de PI-3K, PLC-γ, Ras-Raf-MAPK. SV40LT, HPVE6, E1B-55K/E4-orf6 se lient à p53 et l'inactivent. Il est à noter que la protéine p53 a été identifiée pour la première fois comme une protéine qui se lie à SV40LT. Ces protéines virales forment des complexes à plusieurs unités avec p53 et cause sa dégradation. Par exemple les complexes E1B et E4-orf6, comprennent la Culline 5, la protéine avec un doigt RING, Rbx-1 et l'Elongine (164). Le HPV E6 utilise E6AP (*E6-associated protein*) pour induire la dégradation de p53. Cependant, aucune des protéines précoces (PyS, L and MT) du virus du polyome ne lie p53. Le PyMT induit l'ARF qui active p53. Toutefois, p53 n'augmente pas en présence de PyST.

L'ARF lie MDM2 et la séquestre dans le nucléole. Il inhibe également sa capacité à dégrader p53 indirectement. PyST inhibe l'ARF via son domaine qui se fixe avec PP2A. PP2A existe sous forme de complexes hétérotrimériques composés des unités A, B et C. PyST déplace la sous-unité B et modifie la spécificité du substrat de la phosphatase. La phosphatase pourrait déphosphoryler p53 et/ou MDM2 et affecter leur in

teraction. La cycline G recrute PP2A pour MDM2 et stimule la destruction de p53 médiée par MDM2 (165, 166). La Figure 17 montre les interactions des protéines virales avec les molécules qui régulent la progression du cycle cellulaire et la réponse au dommage d'ADN.

1.4 La Progression du Cycle Cellulaire dans les Cellules Infectées par les Virus Herpès.

Les herpèsvirus sont de gros virus complexes avec des génomes d'ADN double brin relativement grands (130-235 kb). En raison de leur grand génome, ils peuvent se permettre de coder des gènes nécessaires à la synthèse d'ADN. Ces virus sont moins dépendants des machines de la cellule hôte pour leur réplication comparativement aux petits virus à ADN, qui exigent et maintiennent les cellules infectées en phase S. La particularité de ces virus est leur capacité à entrer en latence et de se cacher dans les cellules de l'hôte. Durant la latence, le génome viral persiste sous forme d'ADN extra-chromosomal (épisome) dans des cellules hôtes avec le minimum d'expression des gènes viraux. Les virus latents s'activent dans un petit sous-groupe des cellules infectées qui supportent la réplication lytique du virus. Ces virus sont capables à la fois d'activer et de bloquer la progression du cycle cellulaire. Il existe des interactions étroites mais complexes entre les virus herpès et les mécanismes de régulation du cycle cellulaire. La Figure 17 montre plusieurs protéines des virus Herpes qui interagissent avec les molécules régulatrices du cycle cellulaires. Il arrive que les infections n'induisent pas l'arrêt des cellules infectées dans une phase précise du cycle cellulaire. Les cellules infectées peuvent sembler plutôt s'arrêter dans une phase particulière, alors qu'elles ont encore certains événements caractéristiques d'une autre phase du cycle cellulaire. En général, le virus vise à changer le microenvironnement interne de la cellule de sorte qu'il favorise sa réplication et son maintien. Certaines protéines virales suscitent l'arrêt du cycle cellulaire alors que d'autres peuvent y activer certaines voies régulatrices; revue dans (40).

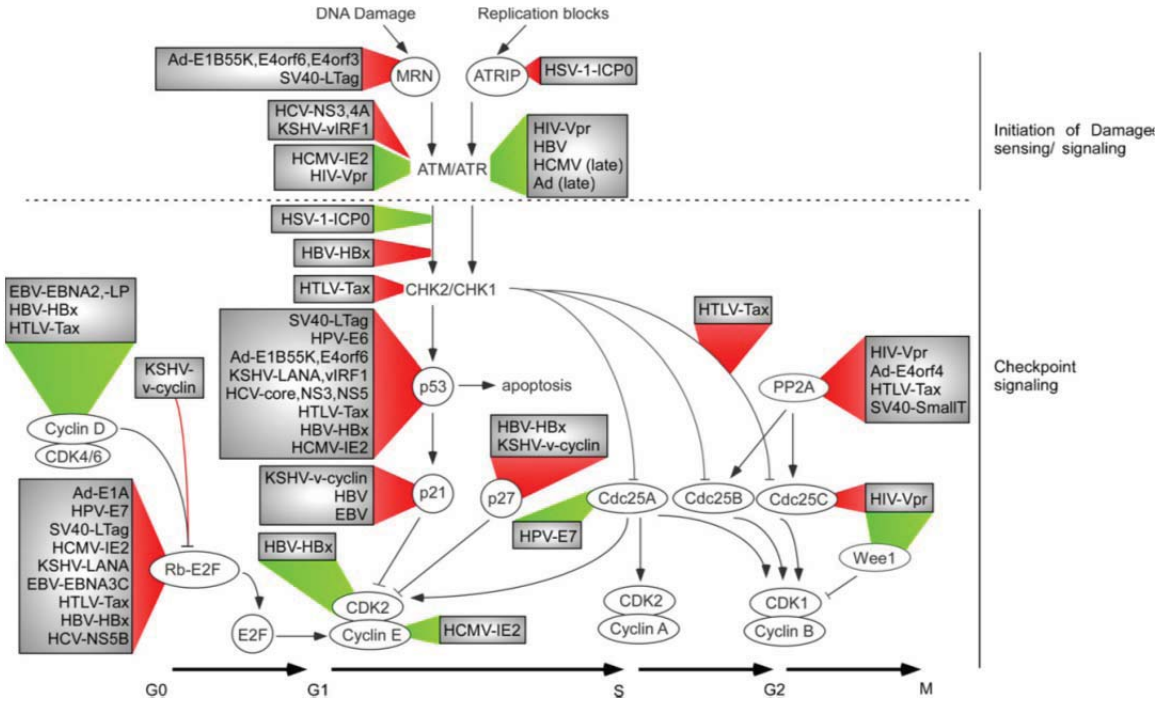


Figure 17. Les interactions des protéines virales avec les molécules régulant la progression du cycle cellulaire et la réponse au dommage d'ADN.

Les cercles et les rectangles gris indiquent les protéines cellulaires et virales, respectivement. Les triangles rouges correspondent aux inactivations, alors que les verts correspondent aux activations. D'après la référence (167).

Il faut noter que dans le cas où la détermination du cycle cellulaire est basée sur la teneur en ADN, il est difficile de distinguer entre un arrêt en S ou en G2 des cellules infectées par ces virus en raison de la présence de l'ADN viral.
Dans ce qui suit, nous avons sélectionné un virus représentatif pour chacune des trois sous-familles des Herpèsvirus et nous discutons la manière dont il régule la progression du cycle cellulaire dans les cellules infectées.

1.4.1 Le Virus Herpès Simplex (HSV-1).

HSV-1 est un membre de la sous-famille des α-Herpesvirinae qui infecte l'homme de façon ubiquitaire. Les infections virales primaires chez les humains surviennent en bas âge. Ces infections sont souvent bénignes et peuvent se manifester sans préavis. Cependant, les hôtes infectés ne parviendront jamais à s'en débarrasser et ils en resteront porteurs tout le reste de leurs vies (168). Ce virus migre à partir des terminaisons nerveuses de la muqueuse buccale et de la peau du visage et reste latent dans les ganglions de la racine dorsale du nerf trijumeau. Les infections latentes se réactivent sous un stress, une immunosuppression, un traumatisme physique ou encore suite à l'exposition aux rayonnements UV (169). La réactivation de ces infections se manifeste par l'apparition de cloques douloureuse appelées bouton de fièvre ou « feu sauvage » au niveau des jonctions de la cutanéo-muqueuse des lèvres. Ces boutons de fièvre sont souvent une source de malaise considérable chez les humains. Le virus peut infecter également la cornée entraînant une kératite. Rarement, les infections chez les enfants provoquent une encéphalite. Les personnes immunocompétentes ayant des défectuosités inconnues dans le système immunitaire peuvent aussi souffrir d'encéphalite causée par HSV-1. Il est fréquent que ce virus occasionne des encéphalites sporadiques infectieuses chez l'homme d'apparence saine (168). La plupart des médicaments anti-HSV qui ont été développés ciblent principalement l'ADN polymérase virale. Toutefois, l'apparition de souches virales résistantes est un problème croissant qui fait appel à la nécessité de développer de nouveaux médicaments; revue dans (170). HSV-1 est capable d'infecter des cellules quiescentes en G0 et de stimuler la progression de leur cycle cellulaire vers la transition G1/S. Cependant, il inhibe la synthèse d'ADN cellulaire. Même les particules virales qui n'ont pas leur contenu en ADN sont aussi capables de bloquer efficacement la synthèse d'ADN cellulaire. Ceci suggère l'implication de protéines associées aux virions dans ce processus. Dans l'ensemble, HSV-1 augmente l'activité de cdc2 et diminue celle de CDK2 (171). Le génome viral code pour de nombreuses protéines qui peuvent interagir avec différentes molécules régulatrices du cycle cellulaire. L'infection virale entraîne une accumulation des E2F4 répressives dans les noyaux où elles vont se complexer avec p107 et arrêter les cellules en G1. L'infection

inhibe de nombreux événements cellulaires liés à la phase G1, comme, l'induction des cyclines D1 et D3, les activités de cycline D/CDK4, cycline D/CDK6 et cycline E/CDK2 ainsi que la phosphorylation de pRb (172). Grace à ces événements, HSV-1 empêche les cellules en G1 de traverser le point R et celles en S de terminer le cycle cellulaire. Il induit l'expression de trois protéines virales ICP0, ICP4 et ICP27 (*infected cell protein 0, 4 et 27*) impliquées dans l'arrêt du cycle cellulaire à la transition G1/S (172-175).

Les virus mutants pour ICP27 n'arrivent pas à arrêter les cellules infectées à la phase G1. La protéine virale ICP27 et la protéine vhs (*virion host « shutoff »*) réduisent l'expression des ARNm de la cycline D1 (173). La protéine virale ICP0 est une composante du virion qui joue un rôle dans cet arrêt. Les ICP0 arrêtent les cellules qui sont synchronisées en G1, en G2/M. D'autre part, la protéine induit l'expression et la stabilisation de p53 en la phosphorylant au niveau des résidus Ser-15 et Ser-20, même dans des cellules ATM-négatives (176). ICP0 possède une activité ligase E3-ubiquitine et cible plusieurs protéines cellulaires clés importantes pour une destruction médiée par le protéasome (177, 178). L'infection virale par HSV-1 induit également l'expression de p21, MDM2 et de GADD45 dans les cellules infectées, même si p53 est mutée. Toutefois, le virus augmente les activités des complexes cycline B/CDK1 dans les cellules infectées. Deux inhibiteurs de CDK1, la roscovitine et l'olomoucine, ainsi que des mutants dominants négatifs de la kinase inhibent efficacement la réplication de HSV-1 dans les cellules infectées (179). La protéine virale VP16 nécessite une CDK1 fonctionnelle pour activer la transcription des gènes IE. Les inhibiteurs de la kinase bloquent les transcrits du gène IE. La roscovotine diminue l'activité du promoteur d'ICP0. L'analyse des mutants de HSV-1 qui n'expriment pas les protéines IE ont montré que HSV-1 induit un arrêt du cycle cellulaire indépendamment de p53 et que le blocage mitotique causé par ICP0 est une conséquence directe de sa capacité à agir comme un doigt de zinc « *zinc binding RING finger* » et comme une ligase E3. ICP0 peut interagir avec p53, l'ubiquitiner et dégrader une bonne proportion de ces molécules (180). Par conséquent, cette protéine protège les cellules U2OS de l'apoptose induite par les UV. Lorsque le virus infecte les cellules en G2, elles vont s'arrêter dans un stade de "pseudoprométaphase" en raison de la dégradation des protéines associées au centrosome comme les histones 3-like, CENP-C et CENP-A (*centromeric protein A and C*). La protéine ICP0 induit ces changements (181). Ces histone-like protéines sont associées au centrosome, elles aident à maintenir la structure nucléosomale de l'ADN cellulaire au kinétochore en prométaphase; revue dans (182).
Il faut remarquer que le génome viral est maintenu sous une conformation nucléosomale dans les cellules infectées de façon latente. ICP0 pourrait jouer un rôle dans la rupture de cette structure d'ADN lors de la réactivation du virus. Fait intéressant, ICP0 se lie à l'USP-7 (*ubiquitin-specific protease-7*). Cette interaction empêche l'auto-dégradation d'ICP0 médiée par l'ubiquitine (183, 184). Pour s'activer, ICP0 nécessite une kinase qui est sensible à la roscovotine. La roscovotine inhibe donc la capacité de cette

protéine virale de transactiver ICP-6 (185). Les cdc2 doubles mutants négatifs inhibent également la réplication de HSV en inhibant l'expression d'US11, le gène de latence (γ-2). La roscovotine inhibe aussi la réactivation de HSV de la latence dans les neurones.

Malgré une activité élevée de CDK1, l'infection est accompagnée par la disparition progressive de la cycline A suivie de celle de la cycline B dans les cellules infectées. En outre cdc25C est hyperphosphorylée dans les cellules infectées. Ces changements sont médiés par les produits des gènes viraux UL13 et α-22/US1.5 (171, 186). Le facteur de processivité UL42 de la polymérase virale d'ADN se lie à cdc2 à la place des cyclines et augmente son activité kinase. Le complexe se lie et phosphoryle la topoisomérase IIα de l'ADN. La phosphrylation de la topoisomérase II de l'ADN par l'ICP22 est nécessaire pour son interaction avec le complexe cdc2/UL42. La topoisomérase phosphorylée est requise pour démêler la progéniture d'ADN concatémèrique (186). Vers le stade finale de l'infection, les E2F sont post-translationnellement modifiées ce qui réduit leur capacité de se lier à l'ADN, et leur exclusion des noyaux. Ceci pourrait s'expliquer par une inhibition (*shut off*) des gènes de la phase S à la fin de l'infection (187).

L'ICP0 interagit également avec la cycline D3 et la stabilise.

L'infection virale suscite la réponse aux dommages d'ADN, qui se traduit par le recrutement des protéines cellulaires de la recombinaison cellulaires et leur cheminement vers les sites de réplication virale (188). Elle hyper-phosphoryle et séquestre la protéine RPA (*replication protein A*) endogène dans les compartiments de la réplication virale. RPA est une protéine nucléaire qui se lie à l'ADN simple brin qui génère des signaux de stress génotoxique par la voie ATR. Ceci suggère que le virus prévient une réponse normale de signalisation des ATR. La protéine ATRIP (*ATR-interacting protein*) recrute l'ATR et le γ-H2AX lors d'une réponse à l'endommagement. Cette dernière protéine est un marqueur de cassures d'ADN double brin. Ces deux protéines s'accumulent là où se trouvent les sites de lésion d'ADN au début d'un stress génotoxique. L'infection par HSV empêche le recrutement des facteurs de réparation pour les lésions d'ADN. Elle découple spatialement l'ATR de l'ATRIP, et séquestre l'ATRIP et la RPA endogènes hyperphosphorylée dans les domaines nucléaires induits par le virus (ND-10).

Ce type de séquestration et de découplage peuvent être fait par ICP0 seul (189). L'utilisation des virus ICP0 null ou de promoteur mutant, a permis de conclure qu'ICP0 n'est pas capable d'initier la réactivation virale dans les neurones lors de la latence (190). Par conséquent ICPO n'est pas une composante responsable de la sortie de la latence vers la réactivation, mais elle est importante pour la réactivation. La protéine induit l'arrêt en G2/M qui dépend de l'ATR-CHK2 et la phosphorylation de cdc25C. Un déficit en CHK-2 diminue la croissance des virus de type sauvage. Cela active une réponse aux dommages d'ADN pour arrêter les cellules en G2/M et incite la réplication du virus.

ICP22 médie la dégradation des cyclines A, B et des formes inactives de cdc2. Le complexe cdc2/UL42 recrute et phosphoryle la topoisomérase II pour une expression

efficace des gènes viraux γ-2 tels que : UL38, UL41 et US11. Cdc25C supprime normalement deux phosphates inhibiteurs et active cdc2 dans les cellules infectées. ICP22 et US3 kinases phosphorylent cdc25C. Les cdc25C phosphorylées ne peuvent pas activer cdc2 (191). ICP22 est une protéine régulatrice codée par le gène α-22. ICP22 et UL13 médient la phosphorylation de l'ARN polymerase II dans son extrémité C-terminale. ICP-22 interagit avec CDK9 et le complexe phosphoryle l'extrémité C-terminale de l'ARN polymérase II d'une manière dépendante d'US3. L'US3 agit de façon spécifique aux cellules (192).

La protéine virale pré-précoce US34.5 antagonise PKR-A *((ds) RNA-dependent protein kinase A)* en déphosphorylant la traduction eucaryotique du facteur d'initiation eIF-2a (193). Lors de l'infection, il y a activation de PKR, sauf que le virus l'inactive. L'infection phosphoryle et active également PERK (*PKR-related* endoplasmic reticulum *kinase*). La cycloheximide mais pas le PPA (*phosphonoacetic acid*) bloque cette activation. La protéine US34.5 bloque l'activation des deux PKR et PERK par déphosphorylation d'eIF2A. La phosphorylation de PERK est une marque de stress du RE.

ICP0 est un gène IE et un transactivateur de promiscuité. Il agit en activant NF-kB via la dégradation d'IkBα et en dissociant HDAC 1 et 2 du co-répresseur transcriptionnel Co-REST/REST (194, 195). ICP0 est phosphorylé par la kinase UL13 ainsi que d'autres kinases virales dans les cellules infectées. ICP22 est surproduit en l'absence d'UL13. ICP0 est aussi une ligase E3, situé dans le locus associé à la latence. Il inhibe les effets antiviraux de l'activation des STAT-1 induite par l'IFN-γ. La première série de gènes (ICP0, ICP4) est activée par la protéine associée au virion VP-16. Ce sont les protéines produites de façon précoces (les gènes IE comme ICP0) qui induisent l'apoptose et non pas l'attachement viral, l'entrée et la fusion membranaire, alors que les protéines virales tardives préviennent cette apoptose. Ainsi, la cycloheximide induit l'apoptose dans les cellules épithéliales humaines HEp-2 infectées par le virus. ICP22, empêche aussi la mort cellulaire. Par contre, les deux protéines gD ou gJ bloquent également l'apoptose lorsqu'elles sont administrées en transe dans les cellules SK-N-SH (196, 197). Le virus empêche l'apoptose qu'elle soit causée par certaines protéines virales, ou par des stimuli externes (TNF-α), un anticorps agoniste anti-Fas, la céramide C2, un choc osmotique avec du sorbitol, ou par un choc thermique. Toutefois, ceci dépend du type cellulaire. Très tôt dans l'infection, le virus induit une apoptose qui ne nécessite pas l'expression des gènes viraux. Les virus mutants pour les gènes Alfa-4 et US3 induisent l'apoptose mais pas ceux de type sauvage. Alfa-4 code pour une protéine régulatrice majeure et US3 code pour une kinase. Le virus de type sauvage protège les cellules SK-N-SH sub-confluentes et non pas les cellules HELA de l'apoptose due au TNF-α, anti-Fas, céramide C2 et au choc thermique. Ce virus ne protège pas les cellules de type confluentes du choc osmotique. Les inhibiteurs de caspase bloquent l'apoptose induite par le sorbitol et la céramide, mais pas celle causée par les virus mutants. Ainsi, d'un côté, le virus déclenche l'apoptose en

déjouant de multiples points de contrôle métaboliques, et d'un autre côté, il a développé des mécanismes pour inhiber l'apoptose causée par des stimuli internes et certains externes (198).
Dans certains types de cellules, ICP22 est requis pour l'expression des gènes tardifs (γ 2) en raison de l'altération des cyclines de la phase S. Il induit, active ou réprime l'activité des protéines cellulaires. Il dérégule le cycle cellulaire lors de l'infection des cellules en phase S permissive (199).
HSV-2 est un virus étroitement apparenté à HSV-1 qui infecte une lignée cellulaire de reins de singe vert africain CV-1 en G0. Il induit l'expression de la cycline A et la stimulation de CDK2, 8 à 10 fois deux heures après infection, ensuite il les diminue 16-24 heures après infection (200). L'activation transitoire de CDK2 provoque la phosphorylation de pRB. Selon cette étude, l'infection n'a aucun effet sur l'activité de CDK4, mais il entraine une diminution de l'expression et de l'activation de CDK1 (due à la phosphorylation de la tyrosine). De plus, les auteurs n'ont pas observé une progression du cycle cellulaire dans les cellules infectées. Ils ont donc conclu que HSV-2 n'entraine pas la progression du cycle cellulaire, mais qu'il a juste besoin de certains événements se produisant en phase S.

Certaines fonctions sont conservées par exemple les homologues d'UL24 en version alfa, bêta et gamma inactivent cycline B/CDK2, arrêtent les cellules en G2 et induisent l'apoptose chez la souris et les cellules humaines; Orf 10 du MGH 68, UL-24 de HSV-1'ORF 20 de KSHV, UL -76 de HCMV; (201); UL97 miment cyclin B /CDK1,
phosphorylent et dégradent les lamines nucléaires A et C nécessaires à l'évacuation nucléaires (202).

1.4.2 Le Cytomegalovirus Humain (HCMV).

Parmi tous les herpèsvirus humains, le HCMV, désigné par HHV-5, a le plus grand génome (environ 235 kb); revue dans (203). Approximativement 50 à 100% des adultes de différentes populations humaines pourraient être infectés par ce virus. Ces infections sont généralement asymptomatiques chez l'homme immunocompétent. Cependant, il arrive que ces infections engendrent des maladies sévères chez les nouveau-nés et les personnes immunodéprimées, particulièrement chez les patients ayant subits des transplantations d'organes et de moelle osseuse, ainsi que ceux atteint du SIDA. Les infections prénatales des bébés peuvent se produire via le placenta et entraîner des anomalies congénitales comme une perte d'audition, un retard mental ou encore une mortalité. Ce virus est la cause principale des maladies congénitales chez les nouveau-nés dans les pays développés (204). L'infection par HCMV provoque souvent la rétinite, l'entérocolite et la gastro-

entérite chez les patients atteints du SIDA. Le virus reste latent chez les personnes en bonne santé mais il peut se réactiver suite à un stress, à une inflammation ou une immunosuppression. TNF-α et les prostaglandines pro-inflammatoires libérés sous ses conditions induisent l'expression des gènes viraux IE et déclenchent la réplication virale (203).

HCMV infecte une large gamme de cellules humaines y compris les fibroblastes, les cellules endothéliales, les cellules vasculo-musculaires lisses et les macrophages dans lesquelles il subit la réplication lytique. Souvent, suite à l'infection, les cellules s'élargissent, c'est la cytomégalie qui caractérise le virus et qui est à l'origine de son appellation. Dans les cellules des lignées myéloïdes, l'infection persiste sous la forme latente. Le virus se réactive alors lorsque ces cellules se différencient en macrophages et en cellules dendritiques. HCMV régule le programme transcriptionnel des cellules infectées, il affecte leurs différenciations et subvertit la progression du cycle cellulaire. Ce virus a développé plusieurs mécanismes pour détourner le cycle cellulaire à son avantage. Ces mécanismes convergent généralement vers la voie pRB-E2F. Globalement, le virus arrête les cellules infectées à la transition G1/S (205). Bien que les cellules soient arrêtées à la fin de G1, elles montrent de nombreux événements caractéristiques de la phase S. La Figure 18 montre plusieurs protéines virales du HCMV qui interagissent avec les molécules régulant la progression de cycle cellulaire et la réponse au dommage d'ADN. Toutefois, il n'y a pas de synthèse d'ADN cellulaire (90, 206). HCMV peut se répliquer dans les cellules épithéliales et endothéliales qui ont entreprit leur différenciation terminale. L'infection est capable d'induire la prolifération de ces cellules avec succès. Ainsi, le virus est potentiellement oncogénique et il est même impliqué dans le développement de différents cancers chez les humains; revue dans (207).

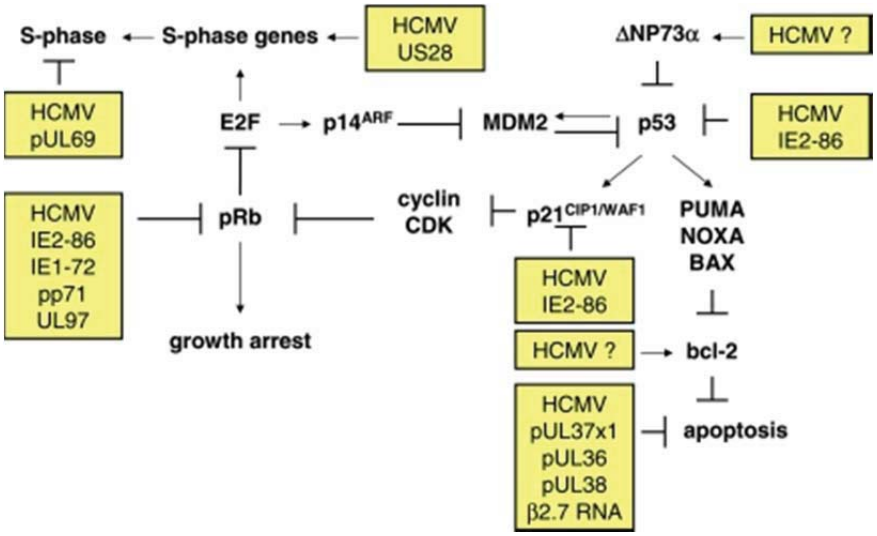


Figure 18. Les protéines du HCMV et leurs interactions avec les molécules régulatrices du cycle cellulaire.

Voir le texte pour les détails. D'après la référence (207).

Lorsque des fibroblastes humains sont infectés en G0 ou en G1, le virus subit une réplication sans que la synthèse d'ADN cellulaire ne se produise. La réplication virale est retardée une fois que ces cellules sont synchronisées en S avant l'infection. Les cellules infectées passent à travers les phases S et M, et endurent l'arrêt dans la phase S suivante. Comme mentionné ci-dessus; l'infection semble provoquer l'arrêt en S, mais la synthèse d'ADN cellulaire ne se produit pas. En fait, le virus n'a pas besoin de la réplication de son ADN, ni de l'expression des gènes tardifs ou γ pour moduler la progression du cycle cellulaire dans les cellules infectées (208). Il a été démontré que deux protéines virales pré-précoces (IE : *Immediate early*); 72 kD IE1, et 86 kD IE2, interagissent avec E2F ainsi que d'autres facteurs de transcription, et qu'elles améliorent la transcription des promoteurs E2F-dépendants, tel que le promoteur du gène DHFR (dihydrofolate réductase) (209, 210). La DHFR est nécessaire pour la biosynthèse des purines, de la thymidine et de la glycine. Une protéine associée aux virions pp71 entre dans le noyau et induit l'expression des gènes IE. Il a été démontré que la protéine virale se lie et dégrade pRB et d'autres protéines de poche via la dégradation protéasomale (211). Une autre protéine associée aux virions, pUL69, induit l'arrêt du cycle cellulaire en G1 dans les cellules humaines (212). Trois protéines virales, pp71, pUL69 et IE1 (ou IE72) créent des conditions simulant la phase S pour faire bénéficier le virus mais ne permettant pas la synthèse d'ADN cellulaire. UL69 induit un arrêt en G1 lorsqu'elle est introduite dans les cellules en réplication. Deux protéines virales pré-précoces IE1 (ou IE72) et IE2 (ou IE86) agissent en tant que transactivateurs viraux et jouent un rôle important au niveau des promoteurs activateurs viraux et cellulaires. Il a été démontré qu'IE86 se lie à l'ADN et qu'elle est indispensable à la réplication virale. IE86 et IE72 ciblent les protéines pRb et p107, respectivement, et les dégradent. IE72 possède une activité kinase intrinsèque. Elle interagit avec E2F1et augmente son activité transcriptionnelle, elle phosphoryle p130 et p107 et les dégrade (90, 206). Ainsi, le virus utilise plusieurs mécanismes pour cibler les protéines de poche et activer E2F. Cette activation prématurée d'E2F pourrait conduire à l'apoptose. L'expression des gènes IE entraine des aberrations chromosomiques. Cette altération de l'ADN mène à l'accumulation de p53. Cette accumulation découle de l'augmentation de la transcription des gènes et de la stabilité accrue de la protéine (213-215). Le virus code pour plusieurs protéines ayant des effets anti-apoptotiques. C'est le cas de la protéine IE86 qui se lie à p21 et p53 et les dégrade. IE72 protège aussi les cellules de l'apoptose (205, 213, 216-218). En outre, l'infection induit delta-nP73α, qui inhibe la mort cellulaire médiée par p53 (142, 206). Deux autres protéines virales pUL36 et pUL37 inhibent également l'apoptose dans les cellules infectées par ce virus. Ainsi, HCMV utilise plusieurs mécanismes pour protéger la progression du cycle cellulaire des cellules infectées du checkpoint induit par p53.

HCMV prévient la synthèse d'ADN par le biais de multiples mécanismes. Il interfère avec l'assemblage des complexes de pré-réplication en empêchant le chargement de MCM sur la

chromatine cellulaire, en diminuant l'expression de Ctd1, en augmentant l'expression de la Geminine et affecte finalement l'acétylation de MCM3. La protéine virale IE86 interagit avec MCM3AP et entraine l'acétylation prématurée de MCM3. Quand elle est acétylée, MCM3 perd sa capacité d'initier la synthèse d'ADN (219). Des études récentes ont montré qu'une autre protéine virale pUL117 vise également les complexes de protéines MCM 2 et 4 pour inhiber la synthèse d'ADN de la cellule hôte (220). L'infection inhibe l'activité hélicase/primase du complexe MCM pour inhiber la synthèse d'ADN. Elle code pour plusieurs analogues de MCM (pUL70, pUL102, pUL105, etc) dans le but de favoriser la synthèse de l'ADN viral. L'infection par HCMV induit l'activation du complexe cycline E/CDK2, et la suppression des CDKI ($p21^{Cip1}$ et $p27^{Kip1}$). HCMV cause également la phosphorylation de pRb. Les complexes induits par le virus se lient à l'ADN à des promoteurs de plusieurs gènes cellulaires, y compris E2F, la cycline A, CDK2 et p107 (221, 222). Il a été démontré que la kinase virale UL97 hyperphosphoryle également pRb, et favorise la progression du cycle cellulaire. Toutefois, la pRB phosphorylée n'est pas dégradée (223, 224). Le maribavir est un médicament relativement nouveau qui inhibe UL97, il est recommandé pour le traitement des infections dues au HCMV et résistantes au gancyclovir (225).

Il est intéressant de savoir que HCMV entraîne une accumulation de la cycline B et l'activation du complexe cycline B/CDK1 (222). Cela se produit suite à la dégradation accrue de Wee-1, au moins en partie. L'activation du complexe indique que le virus ne permet pas aux cellules d'avancer au-delà de G2. L'infection stabilise p53 tout en la séquestrant dans les centres de réplication virale (222). La protéine virale UL76 de HCMV, possède des homologues qui sont conservés chez tous les herpèsvirus, elle inactive cycline B/CDK1, arrête les cellules en G2 et induit l'apoptose dans les cellules humaines et de souris (201). Le virus augmente l'expression de la cycline E, inhibe Cip1 et Kip1 et active le complexe cycline E/CDK2. Le complexe E2F4-DP-1-P130 est spécifique au virus, il se forme dans les cellules infectées et induit la transcription de la cycline E (226). Les protéines virales IE1 et IE2, peuvent induire l'expression de la cycline E dans les cellules eucaryotiques. HCMV induit également l'ornithine décarboxylase, ce qui augmente la synthèse des polyamines (227). Le blocage de l'activité de CDK2 avec la roscovotine; un inhibiteur chimique interfère avec la réplication virale dans les cellules humaines (228). En plus de se lier aux protéines de poche et de les inactiver, IE1-72 active également ATM et cause l'arrêt induit par p53/p21 (229). Toutefois, l'infection stabilise p53 par une dégradation accrue de HDM2 (230). À cet égard, il a été montré qu'IE2 se lie à MDM2 et la dégrade (231). P53 améliore la réplication de HCMV dans les fibroblastes humains (232). L'arrêt de la croissance par IE2 ressemble à la sénescence cellulaire induite par les voies de p16 et p53.

1.4.3 Le Virus Herpes Humain 6 (HHV-6).

Par rapport à d'autres herpèsvirus, on connait relativement peu concernant les effets de HHV-6 sur la progression du cycle cellulaire. Ceci est probablement du à sa cinétique de réplication relativement lente qui complique la manipulation de ce virus. Fait intéressant, le virus augmente la synthèse des protéines des cellules hôtes dans les lymphocytes du sang de cordon. Cela provoque l'élargissement de la taille des cellules (233). Il a été montré que le virus entraine l'accumulation des cellules infectées à la transition G2/M dans les lymphocytes humains du sang de cordon, mais aucune induction de p21 n'a été observée dans ces cellules infectées (234). Une étude ultérieure a montré que la souche PL-1du virus HHV-6B cause l'arrêt des cellules T humaines infectées dans les transitions des phases G1/S et G2/M (235). L'infection augmente progressivement l'expression de p53 ainsi que son activité de lier l'ADN. La majorité des cellules infectées par ce virus ne subissent pas l'apoptose. Les effets induits par le virus sur la progression du cycle cellulaire ne sont pas inhibés par l'acide phosphonoacétique; un inhibiteur chimique de l'ADN polymerase virale. Cela suggère que ce sont les protéines virales IE et/ou E qui induisent ces effets. Les auteurs ont observé une augmentation dans l'expression du gène p53 et de sa phosphorylation sur Ser-15 et Thr-20. Il est à noter que Ser-15 se lie moins à MDM2 et par conséquent échappe à la dégradation causée par MDM2. Le traitement des cellules infectées par la caféine, qui inhibe l'ATM (236), aboli la phosphorylation de p53 sur Ser-15 induite par le virus, mais pas celle sur Thr-20 (235). Dans une autre lignée cellulaire humaine, J-JHAN, le virus induit l'apoptose seulement dans les cellules où il n'y a pas eu d'infection productive et non pas dans celles pourvues d'une infection productive. L'apoptose induite par le virus a été augmentée par le TNF-α exogène (237).

De même pour le virus HHV-7 qui est très étroitement lié à HHV-6, il a été également montré qu'il induit l'arrêt en G2/M d'une lignée de cellules T humaine, Sup-T1. Il cause également la polyploïdie et une augmentation de la taille des cellules (238). Les cellules infectées par ce virus disposent d'une augmentation de l'expression du complexe cycline B/CDK1 ainsi qu'une expression hâtive de la cycline B. Malgré une augmentation de l'expression de ces protéines, l'activité kinase *in vitro* de la cycline B/CDK1 a été diminuée, ce qui est conforme avec la phosphorylation inhibitrice accrue de CDK1 sur Tyr-15. Les auteurs ont également observé une diminution de l'activité phosphatase de cdc25C, une phosphatase qui active CDK1 en supprimant les phosphorylations inhibitrices. Les cellules infectées productivement subissent la mort cellulaire avec des caractéristiques nécrotiques alors que les cellules ayant une infection non productive endurent l'apoptose (239). L'infection a provoqué une régulation positive des TRAIL accompagnée d'une régulation négative du récepteur R1 du TRAIL dans les cellules T CD4 + (240).

1.4.4 Le Virus Epstein-Barr (EBV).

Ce virus infecte les cellules B et les cellules épithéliales du nasopharynx. Il s'agit de l'agent étiologique de la mononucléose infectieuse, une maladie bénigne assez répondue chez les adolescents qui s'accompagne de fièvre, d'une lymphoprolifération et de pharyngite. Les infections primaires surviennent tôt dans l'enfance et elles sont souvent asymptomatiques chez les humains, l'EBV est associé à de nombreux syndromes lymphoprolifératifs ainsi qu'à des tumeurs malignes comme le carcinome du nasopharynx (NPC), le lymphome endémique de Burkitt (BL) (qui se limite géographiquement à la région d'Afrique équatoriale), les différentes formes de lymphomes hodgkiniens et non-hodgkiniens, le syndrome lymphoprolifératif lié au chromosome X, les lymphomes associés au VIH, la leucoplasie orale chevelue, le carcinome gastrique associés à EBV et le lymphome de cellules-T, etc; revue dans (241). En plus de ces maladies, l'EBV a été également associé à plusieurs maladies auto-immunes comme la sclérose en plaques, la polyarthrite rhumatoïde, le lupus érythémateux disséminé, etc (242). EBV immortalise les cellules B humaines et entraine l'établissement des lignées cellulaires lymphoblastoïdes (LCL).

Les LCL indiquent une lymphoprolifération induite par le virus chez les individus immunodéprimés. Chez les personnes en bonne santé infectées par EBV, l'infection virale est limitée aux sous-groupes de cellules B mémoires (243).

À l'intérieur des cellules infectées, l'EBV persiste sous une forme circulaire extrachromosale (épisomale), qui subit la réplication lors de la division cellulaire.

Ce mode d'infection est appelé infection latente. Au cours de la latence, le virus ne se réplique pas, et il exprime un nombre limité de ses gènes.

Les gènes viraux exprimés dans les cellules infectées de façon latente comprennent *l'antigène nucléaire du virus d'Epstein-Barr* (EBNA) -1, 2,3a, 3b, 3c, la protéine EBNA-leader (LP), LMP-1 (*Latent Membrane Protein)*, 2a et 2b.

Contrairement aux protéines EBNA, qui sont exprimées dans le noyau, les produits des trois gènes tardifs, sont exprimés sous forme de glycoprotéines membranaires. En plus de ces gènes, EBV exprime également deux petits transcripts non-polyadenylés, qui ne sont pas traduits. Ces transcrits sont appelés EBERs pour (*EBV-expressed RNA),* ils jouent un rôle dans la régulation de l'expression des gènes viraux et cellulaires. Ils se lient à PKR et inhibent son activité kinase, induisent l'IL-10 lors de la liaison avec le gène-1 induit par l'acide rétinoïque (RIG)-1, et augmentent la survie cellulaire. Ce sont les transcrits les plus abondants lors des infections latentes (244, 245).

Il existe trois profils d'expression des gènes d'EBV au cours de l'infection latente. Pendant la Latence I, seule EBNA-1 est exprimée, tel est le cas du BL. Alors que pour la Latence II, il y a expression de différentes protéines latentes membranaires dont le nombre varie d'une cellule à l'autre. Parmi les protéines exprimées, il y a EBNA-1, LMP-

1, -2A et 2B. Ce modèle d'expression se retrouve dans le NPC et dans le lymphome de Hodgkin EBV positifs. Cependant, au cours de la latence III, toutes les six protéines nucléaires et les trois membranaires d'EBV sont exprimées de la même façon que dans le LCL. BHRF-1, un homologue viral de Bcl-2 (une protéine cellulaire anti-apoptotique) est également exprimé dans le LCL (246).

Certaines cellules du lymphome de Hodgkin EBV-positives n'expriment pas les protéines membranaires latentes; revue dans (247).

Cette expression différentielle des gènes viraux résulte de l'utilisation différentielle de promoteurs viraux alternatifs. En plus des EBERs, la région Bam H1 du génome d'EBV donne lieu à plusieurs ARN non-polyadénylés qui sont traités par la machine cellulaire en micro-(mi)ARN viraux, appelés collectivement BARTS (Bam *H1 A-fragment rightward transcripts*). Bam F H1, une autre région du génome viral, donne également lieu à des transcrits qui sont clivés en miARNs. Les miARN viraux peuvent réguler l'expression de différents gènes viraux et cellulaires (247-249). L'infection des cellules humaines par ce virus induit aussi des changements dans les profils d'expression des microARNs cellulaires. Par exemple, miR-BART 5 inhibe l'expression de PUMA (p*53 upregulated modulator of apoptosis*) (250).

À la réception de stimuli appropriés, les virus qui étaient latents peuvent activer un programme d'expression génique différent et conduisent à la réplication d'ADN viral, à l'assemblage et à la libération du virus. Ce type d'expression de gènes viraux conduit à la mort cellulaire et il est également connu donc étant une infection lytique ou réplicative.

Ce genre d'infection commence par la sur-expression des deux gènes viraux IE, BZLF1 et BRLF1, qui codent respectivement pour le transactivateur Zebra (ZtA) et le transactivateur R(Rta), (241).

L'infection latente d'EBV occasion une prolifération cellulaire. Elle augmente la population des cellules B. D'autre part, l'induction de l'infection lytique aboutit à la production des virus et à la mort cellulaire. Les stimuli qui induisent la différenciation terminale des cellules B ou arrêtent la progression du cycle cellulaire induisent également la réplication du cycle lytique d'EBV. Le virus peut également se répliquer dans les cellules épithéliales complètement différenciées des couches épineuses des muqueuses orales. Le virement de la latence au cycle lytique dans les cellules épithéliales entraine l'arrêt du cycle cellulaire en G0/G1 (251). Comme il est mentionné plus haut, ce virement est médié par le gène viral IE; le transactivateur Zta, ce qui induit la transcription de plusieurs promoteurs viraux impliqués dans les gènes précoces lytiques. Il s'agit d'un facteur de transcription (*basic Leucine zipper*). Ce transactivateur se lie à des éléments du promoteur AP-1 des cellules hôtes (252) et change l'environnement cellulaire en y activant plusieurs gènes. Il interagit avec plusieurs facteurs de transcription, p53 y compris. L'arrêt en G0/G1 induit par Zta est accompagné d'une induction post-transcriptionnelle de $p21^{Cip1}$, $p27^{Kip1}$, p53 ainsi que la hypophosphorylation de pRb. Il semblerait que p53 induit p21 sur ce même modèle. L'inactivation de pRb par

HPV E7 inhibe l'arrêt du cycle cellulaire causé par Zta. Certaines études suggèrent que Zta agit sur le cycle cellulaire indépendamment de son potentiel de transactivation (251). Zta se lie à p53 et l'inactive dans les cellules lymphoïdes. Il n'est pas surprenant que la sur-expression de p53 inhibe les effets de Zta (253). L'enhanceur transcriptionnel CCAAT/EBPα lie Zta, induit l'accumulation de p21 et arrête le cycle cellulaire en G1 au début de l'infection lytique.

L'enhanceur recrute Zta au promoteur p21. Il favorise la différenciation cellulaire, et induit l'arrêt en G1/S en stabilisant p21 (254). Dans un système inductible par la tétracycline, Zta induite l'arrêt en G1, la diminution de l'expression de c-Myc, stabilise CCAAT/ EBP-α, et induit p27 et p53; revue dans (255).

Dans l'ensemble, EBV augmente l'expression de p53 et p21. Le niveau de ces protéines détermine le sort des cellules. Un faible niveau de ces protéines induit l'arrêt en G2/M sans apoptose. Par contre, des taux plus élevés induisent l'arrêt en G1/S et l'apoptose (256).

Les agents qui induisent l'infection lytique causent l'apoptose, alors que les protéines virales lytiques l'inhibent. Deux protéines homologues de Bcl-2 sont exprimées de façon précoce lors de l'infection lytique (BHRF-1 et BRLF-1) et inhibent l'apoptose (257); revue dans (258). LMP-1 est également anti-apoptotique, elle induit deux protéines anti-apoptotiques, A20 et Mcl-1. L'oncoprotéine virale agit comme un CD40 constitutivement actif et elle est considérée comme essentielle à l'immortalisation et aux tumeurs malignes induites par le virus. Son expression active et induit la synthèse d'ADN dans les cellules B primaires (259).

Cette protéine induit l'activation de NFκB et active plusieurs promoteurs cellulaires et viraux. Elle augmente l'expression de la cycline D2 et la phosphorylation de pRb dans les lignées cellulaires BL dépourvues des génomes d'EBV (260). LMP-1 inhibe l'arrêt du cycle cellulaire dû à une privation de sérum en G1, et induit l'expression de la cycline D, de CDK2, d'E2F4 et de p27 (261). Il est à noter que des quantités optimales de p27 sont nécessaires pour la formation des complexes cycline/CDK. L'oncoprotéine virale module l'expression des microARNs cellulaires et influence plusieurs voies de signalisation apoptotiques (262).

LMP-1 peut transformer les fibroblastes des rongeurs qui deviennent oncogèniques chez les souris nudes (263, 264). Les souris transgéniques-LMP développent des tumeurs(265); revue dans (247). L'infection des cellules humaines primaires avec des souches transformantes d'EBV induit l'expression de plusieurs protéines régulatrices du cycle cellulaire. Leur profil d'expression est similaire à celui observé dans les cellules B activées lors d'une réponse immunitaire. Ce profil comprend la phosphorylation de pRb et p107 et par conséquent le relâchement d'E2F.

Le virus augmente l'expression de CDK2, CDK4, CDK6, la cycline D2 et la cycline E (266, 267).

L'infection diminue également l'expression de $p16^{ink4}$, $p27^{kip1}$. Toutefois, le virus augmen-

te l'expression de $p21^{cip1}$ sans toutefois diminuer l'activité kinase de la cycline E/CDK2 (268). Le Figure 19 montre les interactions des protéines virales du cycle latent avec les molécules régulatrices du cycle cellulaire.

EBNA-LP fait partie des protéines exprimées lors de latence. Cette protéine n'est pas essentielle à l'immortalisation mais elle augmente l'efficacité du processus. EBNA-1 est essentielle non seulement pour le maintien du génome viral sous forme épisomale mais aussi pour la désintégration des PML-NB (*Promyelocytic Nuclear Bodies*) via la CK-2 (*Casein kinase*) (269); revue dans (270). EBNA-2 est un antigène nucléaire, qui peut transactiver plusieurs promoteurs cellulaires et viraux.
Il induit l'expression de LMP-1 en supprimant un répresseur nucléaire. Avec EBNA-LP, il peut induire l'expression de la cycline D2 dans les cellules humaines primaires B.
Ces deux protéines induisent la progression de G0 vers G1 et forcent l'entrer des cellules B au repos dans le cycle cellulaire (267).
De la même façon que l'antigène (LT) de SV40, E7 du HPV et E1B d'AdV, EBNA-3C peut transformer des fibroblastes de rat avec Ras activée. Il peut se lier *in vitro* avec pRb et peut dissiper l'inhibition des CDK médiée par $p16^{INK4a}$. EBNA-3C interagit également avec le complexe SCF (skp2) et stabilise l'oncoprotéine c-Myc. Elle cible les effecteurs de CHK2; ATM et ATR et inactive le checkpoint G2/M; revue dans (247). Il a été récemment montré que la protéine se lie à MDM2 et la dé-ubiquitine, elle augmente aussi la dégradation de p53. Elle contribue ainsi, à réduire l'activité transcriptionnelle de p53 (271). La protéine amortit également l'ampleur de la réponse des protéines ainsi que la réponse aux dommages d'ADN (272).

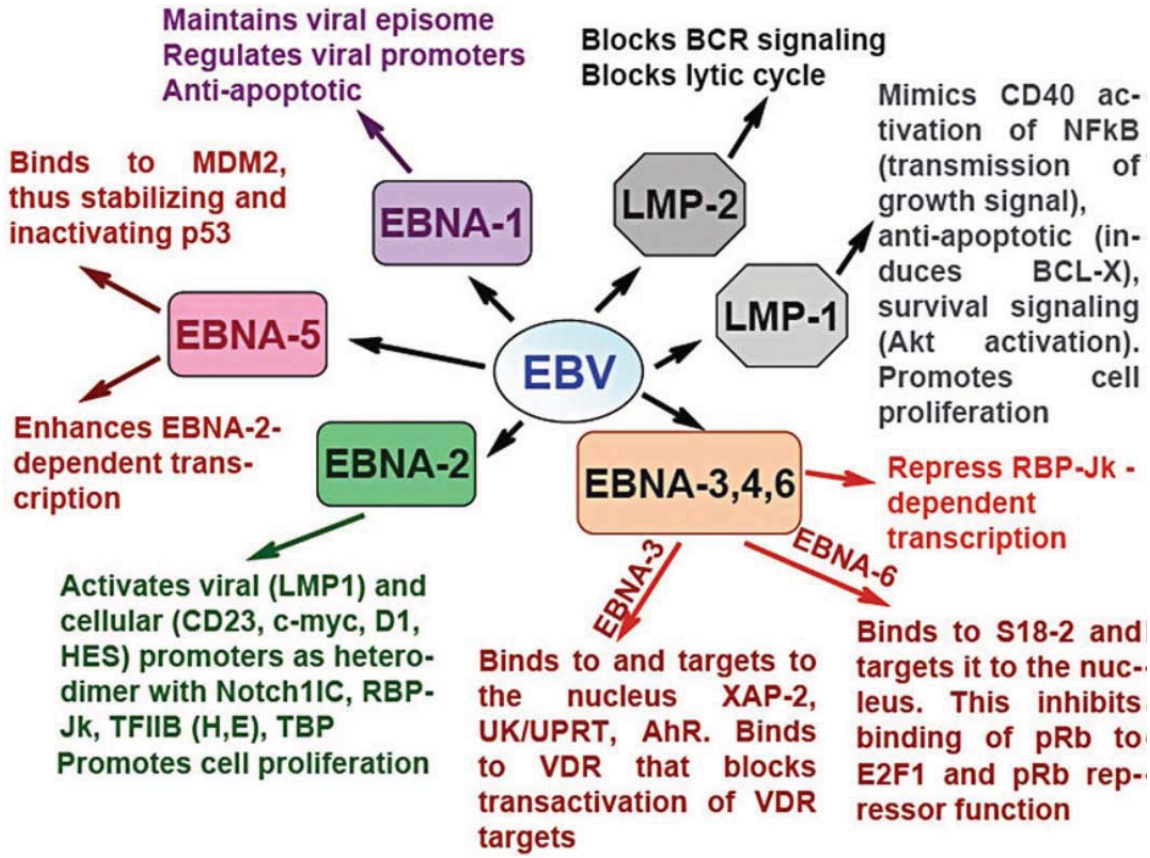


Figure 19: Les interactions entre les protéines du cycle latent d'EBV avec les protéines régulatrices du cycle cellulaire.

Voir le texte pour plus de détails. D'après la référence (273).

Dans les lymphocytes, l'infection latente inactive les points de contrôle du cycle cellulaire. Par conséquent, les lymphocytes infectés de façon latente ne subissent ni l'arrêt en G1 ou en G2/M ni l'apoptose induite par les produits génotoxiques (274). L'abolition des checkpoints ne semble pas être une conséquence due à l'expression LMP-1. L'infection latente inhibe l'arrêt en aval de p53 en empêchant l'inactivation de CDK2. L'infection augmente la dégradation de p21 et par

conséquent, elle n'est plus co-immunoprécipitée avec CDK2 et CDK6, comme il est le cas dans les cellules endommagées (275).

L'infection permet la réplication de l'ADN endommagée dans les cellules infectées. Toutefois, l'arrêt du cycle cellulaire induit par l'irradiation du type gamma n'est pas inhibé par l'infection. L'EBV ne semble pas affecter l'activité de p53 dans les cellules infectées de façon latente. Il a été récemment démontré que le traitement des cellules B transformées par l'EBV avec nutlin-3, un antagoniste de MDM2 entraîne la mort des cellules disposant de p53 de type sauvage (276).

Nutlin-3 stabilise p53 en inhibant son interaction avec MDM2. Toutefois, ceci ne se produit pas dans les cellules p53-mutantes. C'est plutôt l'expression des gènes pro-apoptotiques induite par p53 comme p21, PUMA, MAX, PIG3, NOXA, BAK-1 ainsi qu'une régulation négative de MDMX qui serait à l'origine de la mort de ces cellules. Nutlin-3 agit en synergie avec les inhibiteurs de NF-kB pour induire cette mort (276).

Une étude a démontré que des protéines reliées au cycle cellulaires comme E2F et c-Myc régulent négativement le potentiel de tansactivation de Zta (277). Cela assure l'inhibition de la réplication lytique du virus si les cellules ne sont pas en G0/G1.

L'expression forcée d'E2F et c-Myc inhibe le cycle lytique viral. En outre, Zta lui-même entraine l'arrêt du cycle cellulaire en G0/G1 dans les cellules épithéliales et lymphoïdes. Elle induit également p53 dans les cellules épithéliales. Une régulation négative de c-Myc est un événement clé dans l'initiation du cycle viral lytique. D'autre part, l'infection latente par le virus complèmente c-Myc. Il est à noter que l'activité c-Myc joue un rôle majeur dans le BL associé à EBV. C-Myc se lie à plus de 15% des promoteurs du génome humain (278). Dans les cellules normales, l'expression de c-Myc est strictement réglementée. Ses effets de pro-prolifération (induction des cyclines D et E ainsi que la diminution de p27) sont contrecarrés par sa capacité d'activer ARF-MDM2-P53, pRb et les voies de Bim induisant l'apoptose. Dans le BL, l'EBV semble altérer l'abitité apoptotique de c-Myc. On ne sait toujours rien concernant l'impact de BARF-1, une autre oncoprotéine codée par l'EBV sur le cycle cellulaire ou ses points de contrôle dans les cellules humaines.

2. Hypothèses émises lors de l'étude

1. **Hypothèses concernant HHV-6 et le cycle cellulaire**. Les informations concernant l'effet de HHV-6 sur la progression du cycle cellulaire dans les cellules humaines n'étaient pas encore disponibles lorsque j'ai commencé ce projet. Et même jusqu'à date, les quelques recherches qui se sont penchées sur ce sujet, ont abouti à des résultats contradictoires. Il nous a semblé donc judicieux d'entreprendre ces travaux dans le but d'élucider cette question et de trancher entre les différents résultats fournis par les études ultérieures.

Pour cela nous avancerons donc l'hypothèse suivante; que le virus HHV-6 module et dérègle la progression du cycle cellulaire et qu'il interagit avec les protéines de la machinerie cellulaire pour la détourner en sa faveur.

2. **Hypothèses concernant HHV-6 et l'autophagie**. Nous avons montré dans la première partie que HHV-6 retarde la progression du cycle cellulaire dans les cellules humaines. Comme le cycle cellulaire régule l'autophagie, nous avons cherché à étudier l'impact de l'infection virale sur ce processus cellulaire. À cet égard et jusqu'à présent, aucune étude n'a été réalisée dans le cas de HHV-6. Puisque l'autophagie et la progression du cycle cellulaire se régulent mutuellement, il était intéressant d'étudier l'impact de l'infection virale sur l'autophagie. Nous avons été les premiers à nous pencher sur la question.

Pour cette raison, nous avons émis l'hypothèse d'une éventuelle induction d'une réponse autophagique par HHV-6 dans les cellules infectées et que cette réponse est modulée par le virus en sa faveur.

3. **Hypothèses concernant le rôle de p53 dans la survie cellulaire.** Notre hypothèse de travail était que la protéine p53 exerce un effet inhibiteur sur la réplication de HHV-6 dans les cellules HSB-2 (qui est la lignée cellulaire permissive pour les variantes A du virus) et que sa délétion conduirait à une réplication virale accrue. Pour prouver cette hypothèse et élaborer un meilleur modèle pour la production du virus, nous avons utilisé un siRNA spécifique au gène p53 pour supprimer l'expression de p53 dans les cellules HSB-2. De façon inattendue, nous avons constaté à maintes reprises que la transfection par le siARN, mais pas par le plasmide contrôle, conduisait à la mort cellulaire dans ces cellules. Ces résultats inattendus nous ont conduit à étudier le rôle des niveaux de base de p53 dans la

survie cellulaire. Il est connu que l'induction de p53 peut induire l'arrêt du cycle cellulaire, la sénescence ou la mort cellulaire. Toutefois, ce qui n'est pas connu, est que l'inhibition de l'expression de p53 entraine la mort cellulaire aussi.

À cet égard, mous avons émis l'hypothèse que l'inhibition de l'expression de p53 jouerait un rôle dans la survie des cellules et qu'un niveau basal de p53 est indispensable à leur survie.

3. Objectifs de l'étude

En ce qui concerne notre étude sur HHV-6 et le cycle cellulaire, nos objectifs pour ce projet étaient de:

1. Déterminer l'effet de l'infection des cellules T par HHV-6 sur la progression du cycle cellulaire.
2. Comparer l'expression des gènes et des protéines impliquées dans la régulation du cycle cellulaire dans les cellules T infectées par HH-V6 avec les cellules non infectées.

Les objectifs concernant notre étude sur HHV-6 et l'autophagie.

1. Montrer que l'infection par HHV-6 entraine l'autophagie dans les cellules infectées.
2. Déterminer les effets de la modulation de l'autophagie sur la réplication virale.

Les objectifs destinés à l'étude portant sur p53 étaient de :

1. Déterminer l'impact de l'inhibition de l'expression du gène *p53* sur la réplication de HHV-6. Cependant, l'inhibition de l'expression de ce gène ainsi que l'inactivation de p53 par un inhibiteur pharmacologique a causé la mort cellulaire dans les cellules humaines.

Ce fut tout un résultat inattendu. Par conséquent, nos objectifs sont devenus de caractériser la mort de la cellule et de déterminer son mécanisme

Chapitre 2

Résultats

Résultats 1: Human Herpes Virus-6- induced changes in the cell cycle progression in human T cells

Article 2: Olfa Debbeche, Alexandre Iannello, Suzanne Samarani, Raouf Fetni, Frederique Tihy and Ali Ahmad

Résumé: HHV-6 est un agent pathogène humain ubiquitaire. Ce virus cause la roséole chez les enfants et il a été associé à une variété de syndromes lymphoprolifératifs ainsi qu'à des maladies chroniques chez l'homme. Les effets de l'infection par HHV-6 sur la progression du cycle cellulaire dans les cellules humaines restent encore peu connus. Nous montrons ici que l'infection par HHV-6 ralentit la progression du cycle cellulaire dans une lignée de cellules T humaine, HSB-2, ainsi que dans les cellules T primaires humaines. Le virus retarde leur progression dans la phase G2/M. Nous avons trouvé une augmentation de l'activité kinase du complexe immun cdc2 dans les cellules infectées, malgré une baisse des niveaux de ses partenaires catalytiques, la cycline A et la cycline B. Nos résultats suggèrent qu'une protéine virale, p41 se lie avec cdc2 et augmente son activité kinase. L'infection induit la stabilisation et l'activation de p53 dans les cellules infectées alors que l'expression de son gène cible *p21*, a diminué à des niveaux indétectables dans ces cellules. En outre, le virus a également augmenté l'accumulation des cellules polyploïdes. Ces résultats fournissent des informations nouvelles concernant les effets de HHV-6 sur la progression du cycle cellulaire dans les cellules infectées par ce virus. Ces résultats suggèrent que HHV-6 provoque des perturbations profondes dans la progression normale du cycle cellulaire dans les cellules T humaines.

Human Herpes Virus-6- induced changes in the cell cycle progression in human T cells

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Running Title: HHV-6-induced changes in cell cycle progression

Key Words: cdc-2, cyclin B, HHV-6, p53, p21.

Abstract

HHV-6 is a ubiquitously occurring human pathogen. The virus causes roseola in children and has been associated with a variety of lymphoproliferative disorders and chronic disease conditions in humans. The virus causes immunosuppression and inhibits mitogen-induced proliferation of T cells. However, little is known about the effects of HHV-6 infection on cell cycle progression in human cells. We show here that the viral infection slows cell cycle progression in a human T cell line, HSB-2, as well as in primary human T cells. The virus delays their progression at the G2/M transition. We found an increased kinase activity of the cdc2 immune complex from the infected cells despite decreased levels of its catalytic partners, cyclin A and cyclin B. The latter protein was detected predominantly in the nucleus of the virus-infected cells. The viral protein pU27 was found to associate physically with, and increase kinase activity of, cdc2 in the virus-infected cells. The infection induced stabilization and activation of p53 in the infected cells whereas the p53-inducible genes p21 decreased to undetectable levels in these cells. Furthermore, the virus also increased accumulation2 of polyploid cells. We also documented several changes in the expression of key cytoskeletal proteins in the virus-infected cells. These results provide novel insights concerning the effects of HHV-6 on cell cycle progression in the virus-infected cells. These results suggest that HHV-6 causes profound perturbations in the normal progression of the cell cycle in human T cells.

Key Words: Cell Cycle, Cyclin-Dependent Kinase, HHV-6, p53, p21.

Introduction:

HHV-6 is a lymphotropic β-herpesvirus that was discovered in 1986 (1, 2). The virus occurs ubiquitously in human beings. In most human populations, more than 95% individuals become seropositive for this virus by 2 years of age (reviewed in (3)). Primary HHV-6 infections usually occur in early childhood and cause a mild self-limiting febrile illness called Exanthem Subitum or roseola (4). Occasionally, they may become fatal causing encephalitis and other central nervous system complications (5). In adults, primary infections cause mononucleosis-like illness that may involve hepatitis (6, 7). Once recovered, the infected individuals become life-long carriers of the virus and shed it in their saliva. The infection usually remains latent in healthy individuals but frequently becomes reactivated in immunosuppressed ones, e.g., transplant recipients, AIDS patients, etc, and contributes further towards immunosuppression. HHV-6 is considered as a major cofactor in the development of AIDS in HIV-infected humans (reviewed in (8, 9)). It has also been associated with many other disease conditions, e.g., multiple sclerosis, chronic fatigue syndrome, papular-purpuric "gloves and socks" syndrome, Kikuchi-Fujimoto disease, Rosai-Dorfman disease and a variety of lymphoproliferative disorders including Hodgkin's disease, various types of non-Hodgkin's lymphomas and acute lymphoblastic leukemia (ALL), Burkitt's lymphoma (10-13), (reviewed in (3)). $CD4^+$ T cells are the main target cells of the virus although other cell types, e.g., B cells, Natural Killer (NK) cells, $CD8^+$ T cells, monocyte-macrophages and dendritic cells and can also be infected (14-17). HHV-6 is known to inhibit mitogen-induced proliferation of human T cells, and suppresses the production of IL-2 from them (18, 19). It has also been

documented to cause apoptosis in human cells (20-22). The virus induces host cell protein synthesis, shuts down cellular DNA synthesis, and markedly increases cell size suggesting that it may decouple cell growth from mitosis (23, 24). These observations suggest that this virus may cause changes in the normal progression of cell cycle in human cells. However, a few studies that addressed this issue have reported discordant results with respect to the effects of the viral infection of cell cycle progression (25-28).

The cell (division) cycle comprises a set of tightly regulated and precisely coordinated biochemical events that culminate in the division of a cell into two genetically identical daughter cells. It is arbitrarily divided into four phases. The phases include an S (synthesis) phase, which is characterized by the synthesis and replication of DNA and an M (mitosis) phase in which accurate division of the duplicated chromosomes (DNA) takes place (reviewed in (29, 30)). The S and M phases are separated by two G or "gap" phases: a short G1 phase in which cells prepare themselves for synthesis of their DNA and hence duplication of their chromosomes (S phase); and a G2 phase, which follows synthesis of DNA and precedes actual division of the cell and in which the cell grows to ensure that its resulting daughter cells will receive similar contents. Progression through cell cycle is driven by the activities of cyclin-dependent kinases (CDK), which are essentially serine/threonine kinases (31-33). Each CDK comprises a catalytic subunit (the holoenzyme) and a positive regulatory subunit (a cyclin). Cyclins (A, B, D and E) are proteins, which associate themselves with their specific CDKs, positively regulate their kinase activities. Their production and degradation are

temporally regulated in a very precise manner during different phases of the cell cycle (reviewed in (32)). The CDK4 and CDK6 associate with cyclin D and are active in the G1 phase whereas CDK2 associates with cyclin A and E and is necessary for progression of cell cycle at the G1/S transition (34). The so-called mitotic kinase CDK1 (cdc2) associates with cyclin B and is essential for G2/M transition (35-37). Two families of proteins, Kip and Cip, negatively regulate CDK and its members are called CDK inhibitors (CDKI; (29, 38)). The expression of cyclins and CDKI are regulated by their ubiquitination and degradation (39). Given the catasrotrophic consequences that may result from inaccurate division of cellular DNA and/or from the transfer of damaged DNA to daughter cells, cell cycle regulatory mechanisms are intimately linked to the DNA repair mechanisms. P53 is the pivotal molecule that orchestrates DNA damage response to cell cycle regulation (reviewed in (40-42)). Upon its activation, cells may arrest at G1/S or G2/M transition and repair the damage. Alternately, they may undergo senescence (permanent arrest of cell cycle progression) or undergo apoptosis. DNA checkpoints constitute another layer of surveillance for proper progression of cells through different phases of the cell cycle (43); reviewed in (44, 45). These molecular circuits come into play and arrest cell cycle progression if the cell is not properly prepared for the subsequent phase. Despite these tight regulatory controls, viruses are able to subvert cell cycle to promote their own replication (46, 47); (reviewed in (48)). We report here how HHV-6 infection deregulates normal progression of the cell cycle in a human T cell line.

Materials and Methods:

Virus preparation:

The viral strain of HHV-6 used in this study was GS; an A variant strain isolated from peripheral blood mononuclear cells (PBMC) of AIDS patients in 1986 (1). The viral stock was prepared as described (19). Briefly, 5 x 10^6 HSB-2 cells (see below) were infected with the virus by incubation at 37° C for one hour, washed and incubated at 37° C in a humidified 5% CO_2 atmosphere. When the cells showed cytopathic effects (large balloon-shaped cells), the culture supernatants were collected after centrifugation at 1000g for 15 minutes. The cell pellets were suspended in one ml of the culture medium and subjected to three cycles of freezing and thawing. The lysates were cleared off the particulate matter and added to the culture supernatants obtained from the infected cells. The supernatant was passed through 0.45 μ filters and served as the viral stock. The virus titre of the stock was measured in $TCID_{50}$ as described (49), aliquoted and stored at –80° C. The virus preparation used had a titre of 5 x 10^5 $TCID_{50}$. Similarly obtained supernatant from growing uninfected HSB-2 cells was used for mock infection.

Antibodies and reagents:

The proteasome inhibitor MG-132 was purchased from FMD Biosciences (San Diego, CA). Antibodies were purchased from different sources: for CDK2 (catalogue # sc-163), cdc2 (sc-8395), cyclin A (sc-751), cyclin D (sc-181), cyclin B (sc-245), and retinoblastoma (pRb; sc-50) from Santa Cruz (San Jose, CA), for p53 phosphorylated at Ser^{15} (catalogue # 9286), retinoblastoma (pRb) phosphorylated at Ser 807/811

(catalogue # 9308) and Ser 795 (catalogue # 9301) from Cell Signaling Technology (Mississauga, Canada), for Ser^{10}-phosphorylated H3 were from Upstate (catalogue # 05-598), for securin (catalogue # ab3305) from Abcam, and for α and γ-tubulin (catalogue # T6199 and T6557, respectively) from Sigma-Aldrich. Cytochalasin B (catalogue # C6762), 4, 6-diamidino-2-phenylindole (DAPI; catalogue # D9542), Phalloidin (catalogue # P1951) and PKH-67 (MINI-67; Green Fluorescent Mini Cell Linker kit) were purchased from Sigma-Aldrich. FITC-conjugated anti-mouse IgG (catalogue # 405305) was purchased from Biolegend.

Cell culture and the infection protocol:

An immature human T cell line HSB-2, derived from acute T cell leukemia, was used in this study. It was obtained from the American Type Culture Collection (ATCC, Bethesda, MD) and grown in the medium RPMI-1640 (Gibco/BRL, Burlington, Canada) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2.5 mM L-glutamine and antibiotics (100 units per ml of penicillin from Novapharm, Toronto, Canada; 1 μg per ml of gentamycin from Schering, Pointe Claire, Canada and 2 μg per ml of fungizone from Squibb, Montreal, Canada). The cells were fed twice a week. For infection, $5x10^6$ cell pellets were suspended in one 2 ml of the viral preparation (at 0.2 multiplicity of infection), incubated at 37° C for one hour with intermittent shaking, washed and resuspended in the culture medium containing 5% FBS. The culture flasks were incubated at 37° C in a humidified CO_2 atmosphere. The infected cells usually showed typical cytopathic effects of the infection on day 10 post-infection (pi). At this time point, usually 90% of the cells were infected with HHV-6

as determined by immunofluorescence using a virus-specific monoclonal antibody, 2D6 (data not shown). The antibody neutralizes the virus and recognises the viral glycoprotein complex gp 82/105 (50).

In some experiments, we used human peripheral blood mononuclear cells (PBMC) and purified human CD4+ T cells. For this purpose, the PBMC were obtained from the peripheral blood of adult healthy volunteer donors using Ficoll-Hypaque (Pharmacia, Montreal, Canada) gradients. The cells were activated and expanded by incubating them in the culture medium at 37˚C containing PHA (10 μg per ml; Sigma Aldrich, Montreal, Canada) and IL-2 (100 units per ml; Roche, Mississauga, Canada) for 72 hours. CD4+ T cells were isolated from PBMC by negative selection using a kit (Stem Cell Technology, Canada), as described (51). The purified CD4+ T cell population had less than 5% CD4-negative cells as determined by flow cytometry (data not shown).

Cell cycle analysis:

The percentage of cells in each phase of the cell cycle was determined from their DNA content. For this purpose, 1 x 10^6 cells were washed with PBS, resuspended in a buffer containing sodium citrate (1 mg per ml; pH 7.4) propidium iodide (PI; 50 µg per ml), RNAse (0.05 µg per ml) and Igepal CA-630 (3.0 µl per ml; Sigma, St. Louis, MI). The cells were incubated at room temperature for 30 minutes and then analyzed for PI staining by flow cytometry using CellQuest for data acquisition and Modfit for analysis as described earlier (52).

Detection of apoptosis:

Apoptotic cells were detected by staining them with FITC-conjugated annexin V and propidium iodide (PI) using a commercial kit (Cell Death Detection Kit; BD Pharmingen). The stained cells were analysed by flow cytometry using FACSCalibur and CellQuest Pro software (BD Bioscience). The percentages of cells positive for both annexin V and PI as well as for annexin V alone were considered as undergoing apoptosis.

Western blots:

The expression of different proteins in the infected and mock-infected cells was compared by Western blots as described (53, 54). For this purpose, 5 x 10^6 cells were washed with PBS and lysed in a lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS and sonicated for 15 seconds on ice as described. The lysates were centrifuged at 14000g for 30 minutes at 4° C to remove particulate matter and their protein

concentrations were determined with a commercial kit (Bio-Rad Laboratories, Hercules, CA). Fifty μg of the cell lysate were loaded on 12% SDS-PAGE. The resolved proteins were transferred onto polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA) using a semi-dry transfer system (Bio-Rad). Equal loading of proteins in different lanes of the gel was visually examined on membrane blots by Ponceau S staining. After blocking unbound sites, the blots were incubated with protein specific primary antibodies. Protein bands were revealed on blots by enhanced chemiluminescence by using a commercial kit (Vectastain ABC-AMP; Vector Laboratories,). In some cases, the bands were revealed with alkaline phosphatase (AP)-conjugated secondary antibodies and chromogenic substrates (BCIP and NTB from Promega) following supplier's recommended protocols.

Immunoprecipitation:

In order to perform *in vitro* kinase assays, cyclin B and cdc2 complexes were immunoprecipitated from cell lysates following published protocols (53). Briefly, $5x10^6$ cells were washed with PBS, lysed in RIPA buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 1 mM NaCl, 2 mM DTT, protease and phosphatase inhibitors (2.5 mM PMSF, 0.1 mM sodium orthovanadate, 50 mM NaF, 10μM leupeptin, 100μg per ml aprotinin) on ice. The lysates were clarified by centrifugation at 14000g at 4° C for 20 minutes. They were first precleared with control antibodies and then incubated with protein-specific antibodies for two hours at 4 C with shaking. The immune complexes were removed with protein A and G-conjugated sepharose beads. The beads were washed twice with the lysis buffer (150

mM Tris-HCl with 7.5 pH, 1% sodium deoxycholate, 1% Triton X100, once with 0.5 M lithium chloride and then resuspended in 50 µl of incomplete lysis buffer (50 mM Tris-HCl of pH 7.4, 10 mM $MgCl_2$, and 5mM DTT). The 15 µl of the resuspended beads were used in the cdc2 kinase assay as detailed below.

Treatment of cells with Micrococcal Nuclease (MCN):

Herpesviral DNA is known to be mainly devoid of histones and nucleosomes, especially during the lytic cycle (reviewed in (55)). Since MCN degrades inter-nucleosomal (naked) DNA, we removed DNA from the virus-infected cells by treatment with this nuclease. The nuclease was obtained from Fermentas, Canada Inc. The cells were treated as described (56). Briefly, one million cells were washed with PBS, resuspended in 70% ethanol overnight, washed again with PBS and incubated for 10 minutes at 37˚ C in a solution containing 2 units per ml of the nuclease, 0.05% Triton X100 and 1mg per ml of RNAse 1. The cells were washed with PBS and resuspended in the buffer used for cell cycle analysis as described above.

In vitro kinase assay:

In vitro kinase assays were performed using a commercial kit (SignaTECT cdc2 Protein Assay System; Promega, Madison, WI) following the manufacturer's protocol. The system uses a biotinylated peptide (PKTPKKAKKL) derived from the histone H1. The peptide is preferentially phosphorylated by cdc2 and, therefore, is used to measure cdc2-induced kinase activity; however, other kinases e.g., cdk2 and cdk3 may also phosphorylate it (57). The radiolabled peptide is recovered from the

phosphorylation reaction mixture by a proprietary biotin capture membrane of streptavidin matrix that is provided in the form of 1.25cm x 1.15cm squares. After washing the squares, bound radioactivity was measured by liquid scintillation counting and the kinase activity was determined in picomoles (pmol) of ATP per ug of the protein as detailed in the kit brochure. We used this kinase assay with lysates as well as immunoprecipitated cdc2 and cyclin B from the infected and mock-infected cells.

Fluorescent in situ hybridization (FISH) analysis for ploidy:

Ploidy in mock and HHV-6 infected cells was determined by FISH using a pRb-specific probe (LSI 13 RB1, Vysis; Catalog #32-190001), following the suppliers protocol. Briefly, the slides were pre-treated for 30 minutes in 2x standard saline citrate (SSC) at 37°C then immersed in 70, 85 and 100% ethanol at room temperature for 2 minutes each. After drying, the probes were prepared at room temperature in the Vysis hybridation medium, and then denaturated in 70% formamide in 2X SSC for 2 minutes at 72°C and dehydrated in a cold (-20°C) ethanol series (70, 80, and 100%). After air drying of the slides, the denatured probe (72° for 2 minutes) was added to the slides and covered with a coverslip. The hybridization reaction was carried out in a humidity chamber at 37°C overnight. The post-hybridization washes of the slides were done in pre-warmed (73°C) 0.4x SSC (pH 7.0) solution, for 2 minutes each, and in 2x SSC once at room temperature for 30 seconds, and finally in a bath of PBS buffer at room temperature for 5 minutes. DAPI (2,6-diamidino 2-phenylindol) mixed in anti-fading solution (Vysis) was then added as a counterstain. The slides were examined

under a fluorescent microscope. Ploidy was determined by counting number of FISH spots in nuclei.

The expression profiling of cell cycle regulatory genes:

The expression of different cell cycle regulatory genes was compared between HHV-6- and mock-infected HSB2 cells by using a commercial microarray kit (Super Array Inc, Bethesda MD; Catalog # HS-001). The array contains cDNA fragments from 96 cell cycle regulatory genes, four house-keeping genes (GAPDH, β actin, cyclophilin and the ribosomal protein L13a) as internal controls, and pUC18 DNA as a negative control. The fragments are printed on a 3.8x4.8 cm nylon membrane strips. Complete list of the genes is given at the website www.superarray.com. For synthesizing ^{32}P-labelled cDNA probes, total RNA was prepared from mock and HHV-6-infected HSB2 cells 10 days post infection using Trizol reagent (Invitrogen/Life Technologies). cDNA was synthesized from 5 ug of RNA using Molouny-Murine Leukemia virus reverse transcriptase (M-MLVRT; 50 units per ul; Promega), RNAse inhibitor (RNAsin; Promega) and α-^{32}P-labelled dCTP (10 uCi per ml) using the manufacturer's supplied protocols. The labelled cDNA probes from the infected and mock-infected cells were hybridized to separate arrays, washed and autoradiographed. For each array, the ratios between the transcripts of each of the cell cycle regulatory genes and those of a house keeping gene (GAPDH) were determined by densitometry and compared between the virus-infected and mock-infected cells.

Results

HHV-6 induces cell cycle delays in S and G2/M phases in HSB-2 cells:

In order to determine effects of HHV-6 infection on cell cycle in human T cells, we infected asynchronously growing HSB2 cells with GS strain of the virus and analyzed them for cell cycle at different time points after the infection. The percentages of cells in each phase of the cycle were determined from DNA contents of the cells by flow cytometry. For this purpose, the cells were treated with a buffer containing Propidium iodide (PI) and RNAse as described in the Materials & Methods' section. The cell cycle profiles and the percentages of cells in each phase of the cycle are shown in Figure 1 and Table 1, respectively. Significantly higher percentages (54.93% and 16.33%) of the infected cells accumulated in S and G2/M phases on day 8 postinfection, respectively, as compared with (29.85% and 1.23) of the mock-infected cells. These results clearly show that HHV-6 induces cell cycle arrest/accumulation in S and G2/M phases.

HHV-6 also induces cell cycle delays in S and G2/M phases in primary human PBMC and $CD4^+$ T cells:

Primary human $CD4^+$ T cells are the main targets of HHV-6 in a natural infection in humans. Therefore, we were interested in determining whether the virus also induces cell cycle changes in these cells similar to those seen in HSB-2 cells. To address this issue, we purified human PBMC as well as $CD4^+$ T cells from the peripheral blood of a healthy donor as described in the Materials & Method's section, and infected them in vitro with the virus. The cells were analysed for cell cycle on day 8 post-infection. As shown in Figure 2 and Table 2, the virus-induced changes in the cell cycle in primary

human PBMC and $CD4^+$ T cells were essentially similar to the ones seen in HSB-2 cells.

Treatment with the micrococcal DNAse shows accumulation of cells in G2/M but not in S phase:

HHV-6 infected cells contain abundant amounts of viral DNA. Since the above-shown analysis for cell cycle is based upon DNA content of the cells, the viral DNA may have confounded the results. To address this concern, we treated mock-infected and the virus-infected cells on day 8 postinfection with MCN after their permeabilization. As shown in Figure 3 and Table 3, the MCN treatment did not affect the cell cycle profile in the mock-infected cells, but the cells in the S phase disappeared in HHV-6 infected cells. These data suggest that HHV-6 induces cell cycle delay in G2/M and the accumulation of the infected cells in the S phase was due to the presence of viral DNA in the virus-infected cells.

HHV-6 infected cells accumulate in G2/M phase of the cell cycle:

In order to determine whether the cells accumulated in the G2/M phase were infected with the virus, we analysed mock-infected and the virus-infected cells for cell cycle analysis and the expression of a late viral antigen (50) by using a specific monoclonal antibody (2D6). As shown in Figure 4, the cells positively stained for the antibody (and infected productively with HHV-6) can be found in all phases of the cell cycle. However, they have clearly accumulated more in the G2/M phase.

HHV-6 decreases cell proliferation in the infected cells:

A delay in cell cycle progression is very likely to decrease cell proliferation in HHV-6-infected cells. To investigate whether cell proliferation decreases in the virus-infected cells, we loaded cells with a cytoplasmic dye PKH-67 and aliquoted them in two groups, which were either mock or HHV-6 infected. The dye gets diluted at each cell division. We measured fluorescence of the stained cells on day 0, 4 and 8 post-infection by flow cytometry. As shown in Figure 5, dye is diluted less at each time point in the virus-infected cells as compared with the mock-infected cells. These data suggest decreased proliferation of the virus-infected cells as compared to the control mock-infected cells.

HHV-6 infected cells undergo apoptosis:

As shown in the Figure 1 for cell cycle analysis, a proportion of the cells become subdiploid especially on day 8 postinfection. These cells may represent apoptotic cells. In order to determine directly, whether HHV-6 infected cells undergo apoptosis, we stained cells for conventional markers of apoptosis, Annexin V and PI, using a commercial kit. When examined longitudinally postinfection after every two days, the virus-infected cells started showing positivity for apoptosis on day 6 and reached peak at day 10. The Figure 6 shows apoptosis in the virus-infected and mock-infected cells on day 10 postinfection, when approximately 22% of the virus-infected cells became apoptotic (positive for both Annexin V and PI). At this time point, the virus-infected cells also showed cytopathic effects. It is also clear from these data that almost all the virus-infected cells show enhanced staining for PI suggesting compromised integrity of their plasma membranes.

Phosphorylation of H3 at serine 10 in HHV-6 infected cells:

The phosphorylation of Histone H3 at serine 10 in its amino terminal tail occurs late in G2 at G2/M transition and is considered a hall mark of the cells in the early M phase of the cell cycle (58, 59); (reviewed in (60)). Using a mAb specific for H3 phosphorylated at serine 10 (Catalog # 05-598; Upstate), we determined the extent of H3 phosphorylation in HHV-6 infected cells vis-à-vis mock-infected cells. As a positive control for detecting cells at the M phase, we treated cells with Nocodazole. The drug increases intrinsic GTPses activity of tubulin, inhibits polymerization of microtubules and arrests cells in the G2/M phase (61, 62). Typical results from three different experiments are shown in Figure 7. It is clear from the data that the number of cells in the M phase increases in the Nocodazole-treated mock-infected cells. The virus does not increase the cells in the M phase despite increased expression of the phosphorylated H3. Furthermore, it reduces the accumulation of Nocodazole-treated cells in the M phase. This is likely to happen due to delay of the virus-infected nocodazole-treated cells in G2.

Effect of HHV-6-infection on the expression of cell cycle regulatory proteins:

We determined the expression of several cell cycle regulatory proteins (cyclin A, cyclin B, cdc2, cdk2 and p53) by Western blots using specific antibodies. As shown in Figure 8A, the expression of cyclin A, cyclin B and cdc2 decreased on day 6 and 8 postinfection. No significant changes were observed in the expression of cdk2, whereas a marked increase was observed in the expression of p53 in the virus-infected

cells. The increase was clearly evident as early as 48 hours after the infection and progressively increased with time. Since this kinetic analysis of the cell cycle regulatory proteins showed that the virus induced changes were pronounced when the infected cells start showing cytopathic effects (day 8 and beyond), we determined the expression of other cell cycle regulatory proteins at day 8 post infection. As shown in Figure 8B, despite a decrease in the expression of total cdc2, the expression of inactivated cdc2 (phosphorylated at Tyr 15) was increased in the virus-infected cells on day 8 postinfection. Similarly, despite observing no significant change in the expression of total pRb between the mock-infected and HHV-6-infected cells, marked increases in the expression of this protein phosphorylated at Serine 811 and Serine 795 were observed in the virus-infected cells. Furthermore, despite accumulation of total p53 in the virus-infected cells, the expression of activated p53 (phosphorylated at residue Serine 15) was significantly reduced in the virus-infected cells (Figure 8B). Similar results were observed when Western blots were performed on day 2, 4 and 6 postinfection (data not shown). *P21* gene is one of the main transcriptional targets of p53, and is usually induced in cells in response to p53 stabilization and activation. Since p53 accumulated in HHV-6-infected cells but the amount of transcriptionally active p53 (phosphorylated at Serine 15) was reduced in these cells, we sought to determine the expression of p21 in the infected cells. As shown in the Figure 8B, p21 was not detectable in the infected cells on day 8 post-infection, a time point when p53 is maximally expressed in the virus-infected cells. These results show that although HHV-6 infection induces stabilization of p53 in the infected cells, the stabilized p53 is transcriptionally inactive and is unable to induce expression of one of its main target

genes. We further sought to determine whether p21 was expressed but was degraded in the virus-infected cells. As shown in Figure 8C, p21 became visible in the virus-infected cells when they were treated with a proteasomal inhibitor, however, its expression was still lower than that observed in the mock-infected proteasome-inhibitor treated cells (Figure 8C; compare lane 2 with 4). These data suggest that p21 is still produced in the HHV-6-infected cells, albeit at much reduced levels.

DNA synthesis decreases in HHV-6-infected cells:

In order to determine the effect of HHV-6 infection on DNA synthesis, we measured DNA synthesis by BrdU incorporation for 6 hours. Then, the cells were stained intracellularly with PI and analysed for BrdU expression and cell cycle. The results are shown in Figure 9. The virus infected cells on day 8 post-infection incorporated markedly decreased amounts of BrdU. This suggests that the cells synthesize little DNA at this time point in infection. However, the cells with more DNA accumulate in when they are infected with HHV-6. Most probably, this represents DNA from the replicated virus.

Effect of HHV-6 on the cdc2-associated kinase activity:

Since cyclin B and cyclin A act as catalytic partners of cdc2, decreases in the expression of these cyclins may have affected its kinase activity in the infected cells. In order to investigate this, we determined kinase activity of cell lysates as well as of immunoprecipitated cdc2, cyclin B and cyclin A complexes from the infected and mock-infected cells using a commercial kit as detailed in the Materials and Methods.

As shown in Table 4, the kinase activities of the cell lysates and immnunoprecipitated cdc2 complexes were unexpectedly increased in HHV-6-infected cells as compared to the mock-infected cells. The enzymatic activities of both cyclin A and cyclin B complexes immunoprecipitated from the infected cell lysates were, however, markedly reduced as compared to the mock-infected cells. These results suggest that despite decreases in the expression of its positive regulatory subunits, the kinase activity of cdc2 is increased in the HHV-6-infected HSB-2 cells.

HHV-6 increases ploidy in the infected cells:

In order to determine the effects of HHV-6 infection on cell ploidy as well as to directly count cells in different phases of the cell cycle, we performed FISH analysis using a pRb-specific probe. The cells were used in the analysis with and without prior treatment with Nocodazole (5 ug per ml) for 8 hours. The cells in G1, S, M and G2 were counted and their ploidy was determined under a fluorescent microscope. The results are shown in Figure 10 and Table 5. The infection clearly causes accumulation of the infected cells in G2 and increases cell ploidy.

HHV-6 U27 protein associates with cdc2 and shows kinase activity:

Our in vitro kinase assays showed that the kinase activities of cyclin A and cyclin B immunoprecipitates were decreased but that of cdc2 immunoprecipitate was increased in HHV-6 infected cells. Furthermore, we observed enhanced expression of inactivated cdc2 (phosphorylated at the residue Tyr15) in the virus-infected cells. These observations suggested that the kinase may have bound a novel catalytic partner

in the infected cells. In this regard, we took cues from published work on HSV-1's ORF UL-42, which encodes a protein called DNA Polymerase Processivity Factor (DPPF). The viral DPPF complexes with cellular cdc2 and enhances its kinase activity via a degenerate cyclin box resembling that of the one present in the Proliferating Cell Nuclear Antigen (PCNA; (63, 64)). The viral DPPF immunoprecipitates cdc2 and exhibits cdc2-like activity in i*n vitro* kinase assays. Our computer search revealed that U27 ORF of HHV-6 also encodes a potential DPPF with homology with the DPPF encoded by HSV-1. The HHV-6 ORF U27 encodes a viral antigen, p41, with early late kinetics. The viral antigen has been characterized earlier (65, 66). Therefore, we decided to investigate whether HHV-6 encoded DPPF (p41) also complexes with the cellular cdc2 and affects its kinase activity. To address this issue, we immunoprecipitated cdc2 from HHV-6 infected and mock-infected HSB2 cells using a p41-specific monoclonal antibody (9A5D12; (67) on day 5 post-infection and subjected the immunopreceipitate to Western blot using anti-cdc2 and anti-p41 specific antibodies. As shown in the Figure 11A, the immunoprecipitated cdc2 bound p41 on Western blot in the virus-infected cells but not from the mock-infected cells. Similarly, p41 immunoprecipitates reacted with anti-cdc2 antibodies on Western blots (Figure 11B) from the virus-infected cells but not from the mock-infected cells. As noted above, the cdc2 immunoprecipitates from the virus-infected cells showed higher kinase activity as compared with the mock-infected cells. Furthermore, the p41 immunoprecipitates from the virus-infected and not from the Mock-infected cells also showed kinase activity (0.46 vs 7.55 units, respectively). This kinase activity from the virus-infected cells was higher than the cyclin B immunoprecipitates from both mock-

infected and the virus-infected cells Table 4. Taken together, these data suggest that p41 binds cdc2 in HHV-6-infected cells and increases its kinase activity.

HHV-6-induced changes in the expression of cell cycle regulatory genes:

In order to investigate the effects of the viral infection on the expression of different cell cycle regulatory genes, we used a DNA microarray as described in the Materials & Methods section. The genes showing two-fold more or less expression in the virus-infected cells relative to that of the mock-infected cells are listed in Table 6. It is evident from this Table that the expression of the genes for *p53*, Ki67, E2F5, ubiquitin activating enzyme E1, ubiquitin ligase E3A, and c-Abl tyrosine kinase increases and that of the genes for the small ubiquitin-like modifier (SUMO)-1, ubiquitin conjugase E2, Nedd 8, cdc34, cyclin D3, cyclin E2, bcl-2, bcl-2-associated protein X (Bax), p53-inducible genes GADD45A and *p21*, Ataxia Telangiectasia mutant (ATM) and the breast cancer early onset gene (BRCA1) decrease in HHV-6-infected cells on day 10 post-infection. It is noteworthy that despite increases in the expression of *p53* transcripts, the expression of its target genes (*p21* and GADD45A) decreases.

HHV-6 causes localization of cyclin B to the nuclei:

Because of the important role played by cyclin B in cell cycle progression in late G2 and M phases, we determined expression of the cyclin in the virus-infected cells on day 8 post-infection by flow cytometry. As shown in Figure 12A, the expression of the protein was decreased in the virus-infected cells compared with the mock-infected cells. We also stained the cells intracellularly with a PE-conjugated anti-cyclin B

antibody and DAPI and examined them under a confocal microscope. The results are shown in Figure 12B, similar to the Nocodazole-treated cells, the virus induced intranuclear localization of cyclin B. Some virus-infected cells became big with large multi-lobed nuclei. Cyclin B can be clearly seen inside these nuclei (Figure 12B).

Expression of cytoskeletal proteins in HHV-6-infected cells:

Since cytoskeletal proteins, α-tubulin, γ-tubulin and F-actin, play an important role in cell cycle progression, especially in M phase and cytokinesis. We stained the virus-infected cells intracellularly with protein-specific antibodies or Red-Phalloidin (which binds polymerized F-actin). The nuclei were counterstained in the cells with DAPI. The stained cells were examined under a confocal microscope. The images from the microscope are shown in Figure 13. The figures clearly show that the virus-infected cells are usually enlarged with large, sometimes multilobed nuclei, express more perinuclear α-tubulin, γ-tubulin and more F-actin especially between nuclear segments and inside nuclei. A few virus-infected cells in metaphase and cytokinesis could be seen.

Expression of securin decreases in HHV-6-infected cells:

Securin is a component of chromosome passenger complex, and plays an important role in cell division. In order to know the status of its expression in HHV-6-infected cells, we permeabilized the virus-infected and mock-infected cells (on day 8 postinfection), stained them with an FITC-conjugated anti-human securin antibody,

DAPI and Phalloidin and examined them under a confocal microscope. As shown in Figure 14, the expression of securin was markedly reduced in the virus-infected cells.

Discussion:

We have demonstrated here that HHV-6 infection of the human cell line HSB-2 as well as of primary human CD4+ T cells causes a delay in cell cycle progression in S and G2/M phases of the cell cycle. Consequently, increased %ages of the virus-infected cells appear in these phases of the cell cycle. Interestingly, when we treated these cells with Micrococcal Nuclease (MCN), which degrades internucleosomal DNA, they appeared to be delayed in G2/M only. We interpret these results in this way: since the analysis of the cell cycle is based upon DNA contents of the cells, the presence of viral DNA in G1 cells would make them appear in the S phase. Since Herpesviral DNA is not packaged inside cells into nucleosomes (55), MCN can efficiently degrade it. Although MCN will also degrade a fraction of the cellular DNA (internucleosomal), it had no effect on cell cycle profiles in mock-infected cells. Our results suggest that viral DNA may confound the results obtained about cells cycle profiles of virus-infected cells while using DNA-based methods.

Viruses are known to deregulate the cell cycle progression for exploiting host cell factors and nucleotides for their own replication while at the same time preventing replication of the cellular DNA. Therefore, depending upon their needs, different viruses may cause different changes in the cell cycle progression in the infected cells. The lytic infections of human cells with HSV-1, HCMV, and EBV have been shown to arrest these cells in the G1 phase of the cell cycle ((68), reviewed in (48)). HHV-6,

in this regard, differs from these viruses in the sense that its productive infection causes the infected cells to be delayed in the G2/M phase of the cell cycle. It may be argued that the cells used in our study may be inherently unable to undergo arrest in G1 phase of the cell cycle. We investigated this possibility and found that HSB-2 cells are capable of undergoing arrest in G1 phase of the cell cycle upon an appropriate stimulus, e.g., dexamethasone (data not shown). Furthermore, the virus had similar effects on cell cycle profile in primary human CD4+ T cells (Figure 2, Table 2). Earlier studies reported contradictory results with respect to the effect of HHV-6 on cell cycle progression in human cells. A few studies reported that HHV-6 induced arrest in G1 or G1/S in primary human T cells, and glial precursor cells (19, 25, 26). More recently, a study reported the virus-induced arrest in S and G2/M phases in human T cells (28). The authors did not take into consideration the impact of viral DNA in their studies. Had these workers treated the virus-infected cells with MCN, they may have noted the effect of the viral infection on G2/M, and not on S, phase of the cell cycle. Basically our results are in concordance with this report if we ignore our treatment of the cells with MCN. Interestingly, another study also reported that HHV-6 causes cell cycle arrest in G2 phase in human cord blood lymphocytes (27). The workers did not treat the virus-infected cells with any agent to degrade viral DNA. It is possible that HHV-6 replicates less efficiently in these lymphocytes. The viral DNA does not impact cell cycle profile in these cells. Taken together, the studies support a virus-induced delay in G2 phase human cells. Interestingly, HHV-7 that closely resembles HHV-6 also causes cell cycle delays in the G2/M phase of the cell cycle (69).

We have shown here that the kinase activity of cdc2 is increased in the HHV-6-infected HSB-2 cells, despite the fact that the expression of its catalytic partners, cyclin B and cyclin A, were markedly reduced. Furthermore, the kinase activities of the cyclin B and cyclin A immunoprecipitates were also reduced in the virus infected cells. High functional activities of the mitotic kinase in the face of undetectable levels of cyclin B in HHV-6-infected cells raises the question as to the catalytic partner of cdc2 in these cells. Although we observed a decreased expression of cdc2 in the virus-infected cells, the expression of inactive cdc2 (phosphorylated at Try 15) was increased in the virus-infected cells. It is tempting to speculate that the virus may be encoding a protein that acts as a partner for cdc2 and catalyses its kinase activity. This notion is supported by studies on HSV-1 UL42 gene. The gene encodes the DNA polymerase processivity factor (DPPF), which complexes with cdc2 and acts its catalytic partner (63). The DPPF contains a degenerate cyclin box and structurally resembles the proliferating cell nuclear antigen (PCNA), which also binds cdc2. Cdc2 phosphorylates DPPF at a cryptic site near its carboxyl terminus. Furthermore, the immunoprecipitated DPPF from HSV-1-infected cells shows cdc2-like kinase activity in *in vitro* kinase assays. Our computer search has shown that U27 ORF of HHV-6 also encodes a DPPF homologue with potential phosphorylation sites for cdc2 (unpublished data).

The U27 of HHV-6 has been shown to be transcribed with early late kinetics, and encodes a 41 kD protein (P41; (65, 66, 70). Using a P41-specific monoclonal antibody, we showed here that this viral protein co-immunoprecipitates with cdc2 in the HHV-6-infected cells. The vice versa was also true. Furthermore, the P41

immunoprecipitates showed cdc2-like kinase activities *in vitro* kinase assays. Taken together these data suggest that U27 gene product of HHV-6 partners with cdc2 and increases its kinase activity. It is noteworthy that HSV-1-infected human cells also have increased cdc2-associated kinase activity despite disappearance of cyclin A and B (63). However, the two viruses have different effects on cell cycle progression in human cells: HSV-1 arrests cells in G/S, while HHV-6 causes them to delay in G2/M phase. Differences in the effects of other viral proteins between the two viruses may be responsible for these differential effects on cell cycle expression.

We have shown here that the expression of cyclin B becomes reduced and localized to nuclei in the virus-infected cells. Normally, the cyclin is retained in the cytoplasm during interphase by virtue of a cytoplasmic retention signal (CRS) present in the N terminus of the protein. At the beginning of the M phase, the cyclin is phosphorylated and translocated to the nucleus (71). The intranuclear localization of the cyclin in the HHV-6-infected cells is clear evidence that the virus retains cells in this phase. The cyclin is the catalytic partner of the mitotic kinase. The functional activity of this kinase is regulated by the production of cyclin B in the late G2 phase and is maintained throughout the M phase. The kinase is inactivated by degradation of cyclin B just before the onset of anaphase (36, 37). Despite the reduction/nuclear localization of cyclin B, we observed higher cdc2 activities in the infected cells. The kinase activity associated with the cyclin B immune complexes was markedly reduced in the infected cells, which is in accord with the reduction of this cyclin in the infected cells. Since our expression profiling of the cell cycle regulatory genes by the microarray analysis did not show a decrease in the expression of the cyclin B gene in the infected

cells, a marked decrease in the expression of this cyclin in these cells seems to result from its proteasomal degradation. Tightly controlled proteasomal degradation of cyclins and cyclin dependent kinase inhibitors plays a critical role in the normal progression of cell cycle (reviewed in (72, 73)).

The maintenance of high cdc2 activities in HHV-6-infected human cells suggests that this kinase may be playing an important role in the biology of HHV-6. The canonical phosphorylation site of this kinase is known to be S/T-P-X-K/H/R (57). Our computer search has shown that at least 34 HHV-6 encoded proteins may have this motif (data not shown). One or more of these proteins could be potentially targeted and phosphorylated by this kinase and may be important for virus replication. It may be relevant to mention here that 27 orf of HSV-1 can encode proteins with phosphorylation sites for this kinase and, therefore, can potentially be phosphorylaed by cdc2 (74). More importantly, this cdc2-mediated phosphorylation of HSV-1 proteins has been shown to be essential for viral replication. In the cells expressing a dominant negative mutant cdc2, HSV-1 infection failed to express a subset of late viral proteins (74). Furthermore, roscovitine and olomoucine, two specific inhibitors of cdk including cdc2, inhibit HSV-1 replication in vitro (75, 76). As mentioned above, we found 34 orf that could encode proteins with a canonical motif for this kinase. Phosphorylation of one or more of these proteins may be essential for HHV-6 replication in human cells. Clearly, further work is needed to address these issues. Cdc2 or its putative viral partner may serve as target molecules for the development of anti-HHV-6 drugs. It is notable that cdc2 activity in HSV-1-infected human cells is almost the same as in mock-infected cells (63). However, it is much higher in HHV-6-

infected cells as compared to the mock-infected cells. This suggests that cdc2 inhibitors may have wider therapeutic index for HHV-6 infections as compared to HSV-1 ones.

Our Western blot data shows that despite an increased level of expression of total p53 in HHV-6-infected and mock-infected cells, its Ser^{15} phosphorylated protein levels are decreased in the infected cells. *P53* is a tumour suppressor gene that orchestrates DNA damage responses to DNA repair, cell cycle arrest (temporary or permanent), apoptosis and differentiation (reviewed in (31, 40, 41, 44)). About half of all human tumours are mutant for this gene, while the others may be mutant for the genes that activate and/or mediate its responses. In normally replicating cells, p53 protein binds the so-called human double minute (HDM2) protein and is rapidly degraded via proteasomal pathway. Therefore, it has very short half-life (usually in minutes) in these cells. In response to DNA damage or certain viral infections, it is phosphorylated at multiple residues by myriad kinases, which include checkpoint-1 kinase (CHK-1), checkpoint-2 kinase (CHK-2), DNA-dependent protein kinase (DNA-PK) etc (40, 44, 77). Our data show that HHV-6 induces stabilizes this protein in HHV-6-infected cells. Interestingly, our microarray data show that *p53* one of the genes whose transcripts are maximally increased in the virus-infected cells (Table 6). P53 acts as a transcription factor that induces the expression of several target genes by binding to DNA in the promoters of these genes in a sequence-specific manner. The target genes include *p21*, GADD45, Bax, AIP1 (apoptosis inducing protein-1) and HDM2 (78-80). Since HSB-2 cells have a functional *p53* gene (81), an increased expression of the p53-inducible genes and their products in HHV-6 infected cells is expected. However,

we could barely detect p21 in HHV-6-infected cells on Western blots (also see below). These results suggest that p53 protein may be transcriptionally inactivated in HHV-6-infected cells. Indeed, despite its increased expression, we detected decreased amounts of activated p53 (phosphorylated at ser15 residue) in the virus-infected cells. This is supported by the report that *in vitro* HHV-6 orf 1 (an IE viral gene) binds p53 and inhibits its transcriptional activating potential (82). Taken together, these results suggest that HHV-6 stabilizes p53 in the infected cells; however, it also seems to modify its normal transcriptional functions.

P21, a member of the cip family of CDK inhibitors, is the main mediator of the p53-induced cell cycle arrest in G1 phase (29, 38, 44). P21 binds and inactivates G1 and S phase CDK and causes growing cells to arrest in G1 phase of the cell cycle. Interestingly, we did not observe any increase in the expression of p21 protein in HHV-6-infected cells compared to the mock-infected cells. To the contrary, p21 expression was decreased and became undetectable in HHV-6-infected cells. We observed by Western blots that a brief incubation of the infected and mock-infected cells in the culture media containing a proteasome inhibitor restores its expression in the infected cells. Its level of expression, however, remains lower than that of similarly treated mock-infected HSB-2 cells. These observations suggest that HSB-2 cells are inherently capable of expressing p21. However the protein is synthesised less and/or is preferentially degraded in HHV-6-infected cells via proteasomal pathway. Since this inhibitor is the main mediator of p53-induced cell cycle arrest in the G1 phase, (38, 44) its disappearance in HHV-6 infected cells may explain why they do not undergo arrest in G1 phase of the cell cycle. Our microarray data suggest that the

transcription of *p21* gene as well as of another p53-inducible gene, GADD-45, decreases more than two-fold in the virus-infected cells. This is evidence showing that p53 protein, despite being stabilized, is unable to transcribe some of its target genes in the virus-infected cells.

Our results show that HHV-6 induces significant changes in the expression of several cytoskeletal proteins, α-tubulin, γ-tubulin and F-actin, that may delay cell cycle progression in the virus-infected cells. Of these proteins, α-tubulin heterodimerizes with β-tubulin to form microtubules whereas γ-tubulin is found in centrioles and spindle pole bodies, and is required for nucleation and orientation of microtublues. On the other hand, F-actin plays a role in cell movement, cell shape and polarity as well as in cytokinesis (reviewed in (83)). Viruses especially the Herpesviruses reorganize and use these cytoskeletal elements for transport of virions and their capsids inside cytoplasm and nuclei as well as for their export to the cell exterior. It is not surprising that alfa-Herpesviruses induce their polymerization and redistribution in the virus-infected cells ((84, 85); reviewed in (86)). An increased expression of these proteins in the HHV-6-infected cells may be needed for these viral functions. Interestingly, the virus-infected cells expressed drastically decreased expression of securin. The protein levels peak in M phase. They inhibit onset of anaphase by keeping separase inactive until the onset of anaphase. The destruction of securin by APC/C activates separase (39, 87). Furthermore securin also interacts with and regulates p53 (88). A decreased expression of securin in the virus-infected cells may lead to abnormal separation of chromosomes resulting in aneuploidy and polyploidy. It is noteworthy that we

observed increased incidence of these anomalies in the virus-infected cells (Figure 10).

In summary, we have shown here how HHV-6 deregulates cell cycle progression in proliferating human cells. The virus stabilizes p53 but also inhibits its transcriptional activity, at least for p21. Despite decreasing expression of cyclin A and B, the virus increases cdc2 kinase activity. The viral U27 ORF-encoded p41 associates with, and increases, cdc2 kinase activity in the virus-infected cells. The kinase and its viral partner are likely to play important roles in the viral replication and hence represent novel molecular targets for controlling the viral replication.

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Table 1. Percentages of HHV-6- and mock-infected HSB-2 cells in different phases of the cell cycle

D(n) PI	Treatment	G0/G1	S	M
D2	M	70.47	28.25	1.28
	I	54.31	39.03	6.66
D4	M	72.20	26.40	1.40
	I	50.40	36.98	12.47
D8	M	68.92	29.85	1.23
	I	28.75	54.93	16.33

The cell cycle profiles were analysed from cellular DNA contents by flow cytometry after staining with PI, when the infected cells showed cytopathic effects of the infection. The Figure shows %ages of cells in different cell cycle phases. D(n) indicates number of days (n) post-infection. M and I indicate mock and HHV-6 infected cells, respectively.

Table 2. Percentages of HHV-6- and mock-infected PBMC and CD4+ T cells in different phases of the cell cycle

Cell Type	Treatment	G0/G1	S	G2/M
PBMC	M	94.83	0.78	4.38
	I	60.45	12.19	27.35
CD4+ T	M	30.16	54.65	15.20
	I	43.72	27.36	28.92

Human PBMC and CD4+ T cells were obtained from a normal healthy donor, infected in vitro with HHV-6 or mock virus, and examined for their cell cycle profile on day 8 post-infection by flow cytometry after staining with PI. The Figure represents results from one typical experiment and shows %ages of cells in different cell cycle phases. M and I in the Treatment column denote mock and HHV-6 infection, respectively.

Table 3. Percentages of HHV-6- and mock-infected HSB-2 cells in different phases of the cell cycle after treatment with micrococcal nuclease

Treatment	G0/G1	S	M
M	68.63	31.24	0.13
M-D	68.29	30.85	0.86
I	45.14	38.47	16.39
I-D	59.86	8.79	31.35

Mock or HHV-6-infected cells were harvested on day 8 post-infection, treated with the micrococcal nuclease (MCN) and their cell cycle profiles were analysed by flow cytometry after staining with PI. The Figure shows %ages of cells in different cell cycle phases. M and I denote mock infection and HHV-6 infection, respectively.

Table 4. Cdc2 kinase activities of cell lysates and immunoprecipitated protein complexes from HHV-6-infected and mock-infected HSB-2 cells

Treatment	Lysate	cdc2	cyclin B	cyclin A
Mock	4.62	1.14	6.65	7.53
HHV-6	12.74	8.90	1.15	4.81

The kinase activity was measured using a commercial kit (SignaTECT cdc2 protein assay system; Promega) and is expressed in pmol ATP per μg of the protein in the reaction time of 10 minutes. The assay was repeated twice with similar results. The data from an assay are shown.

Table 5: Ploidy in HHV-6 and Mock-infected cells

Ploidy or Phase	Mock	HHV-6
G1	58	31
S	3	3
G2	32	48
M	1-5	3
Triploid	1	6
Tetraploid	1	11

The cells were examined on day 8 post-infection.

Table 6: HHV-6-induced changes in the expression of cell cycle-related genes

Gene	Expression
G1 Phase	
Cdk4	**19.74-**
Cdc7L1 (S. Cerevisiae cdc7-like1)	**13.54-**
Cyclin D1	2.93-
Cyclin D2	3.39-
Cyclin D3	**8.54-**
Cyclin E1	2.15-
Cyclin E2	**18.45-**
Cdki 1A (p^{21}, cip1)	**2.21-**
Cdki 1B (p^{27}, kip1)	6.22-
Cdc34 (ubiquitin conjugase)	2.82-
Cdc37 (S.cerevisiae homolog)	3.22-
E2F4 (p106/p130-binding)	2.52-
E2F5 (p130-binding)	**8.28**-
Cullin 2	2.65+
Cullin 3	**4.07**-
Skp1 (cyclin A/cdk2-associated p19)	2.03-
Skp2 (cyclin A/cdk2-associated p45)	**3.68+**
NEDD8	**5.63-**
P107 (Rb-like)	2.95-
S Phase	
Cdk7 (Xenopus MO15 cdk-activating kinase)	2.42-
Cdk8	5.56-
Cyclin A1	**12.74-**
Cyclin A	2.71-
Cyclin C (G1/S-specific cyclin)	**14.10-**
Cyclin G1	**13.08-**
Cyclin G2	**7.62-**
Cyclin H	5.62-
Cdc6 (S.cerevisiae homolog)	2.50-
Cdc45L (S. Cerevisiae cdc45-Like homolog)	3.13-
Cdc25A	**31.08-**
MCM2 (mitotin)	2.75-
MCM4	3.13-
MCM5 (cdc46)	**9.67-**
KI-67 (antigen identified by mAb ki-67)	**7.99+**
G2 Phase	
Cyclin B1	3.86+
Cyclin B2	3.56+
M Phase	
Cdc2 (p^{34cdc}, cdk1)	5.94-
Cdc16 (S. cerevisiae homolog)	**10.58-**

Cdc2 (p^{34cdc}, cdk1)	2.36-
PRC1 (Protein regulator of cytokinesis1)	4.39-
Rbx1 (Homo sapiens ring-box protein1)	3.57-
MAD2L1 (yeast MAD2 homolog)-like 1	2.86-
DNA Damage Checkpoints	
ATM (Ataxia telangiectasia mutated)	**5.99-**
Bcl-2	2.22-
GADD45A	**10.17-**
P53	**8.57**+
Polyubiquitin	8.76-
RAD17 (S. pombe homolog)	2.89-
RAD9 (S. pombe homolog)	2.19-
RPA3 (Replication protein A3)	**13.55-**
SUMO1 (Human small ubiquitin-related molecule)	4.67-
UBE3 (Ubiquitin ligase E3A)	2.48+

Cdki, Cyclin-dependent kinase inhibitor; MCM, Minichromosome maintenance deficient; NEDD, Neural cell-expressed developmentally down-regulated; V-abi, Abelson murine leukaemia viral oncoprotein. The expression column indicates fold increase (+) or decrease (-)
in the mRNA of the gene the infected cells compared to the mock-infected cells. Only the genes with ≥ two-fold changes in the transcripts are shown here.

Figure Legends:

Figure 1. Cell cycle profile of HHV-6-infected HSB-2 cells. HHV-6-infected, UV-treated and mock-infected cells were analysed for their cell cycle profiles at different time points post-infection. The analysis was performed by flow cytometry using ModFit software after staining with PI. The X-axis indicates DNA contents of the cells in arbitrary PI staining units. The percentages of cells in each phase of the cell cycle are given in Table 1. D (n) indicates the number (n) of days post-infection. Note that a fraction (23.10 %; shown in blue in the Figure) of the virus-infected cells becomes sub-diploid (apoptotic) on day 8 post-infection.

Figure 2. Cell cycle profile of HHV-6-infected human PBMC and CD4+ T cells. The PBMC and CD4+ T cells were 8 days post infection. The acquisition was performed by flow cytometry after staining with PI. The X-axis indicates DNA contents of the cells in arbitrary PI staining units. The percentages of cells in each phase of the cell cycle are given in Table 2.

Figure 3. Cell cycle profile of HHV-6-infected HSB-2 cell with or without treatment with micrococcal nuclease. Mock or HHV-6-infected HSB-2 (day 8 post-infection) were treated or sham-treated with the nuclease, washed with PBS and were analysed for cell cycle by flow cytometry after staining with PI. The analysis was performed by the ModFit software. The X-axis indicates DNA contents of the cells in arbitrary PI staining units. The percentages of cells in each phase of the cell cycle are given in Table 3. DNAse denotes treatment with the micrococcal nuclease.

Figure 4. HHV-6 infected cells are delayed in G2/M. The virus-infected and mock-infected cells were incubated in a buffer containing PI and RNAse. The cells were washed and incubated with a monoclonal antibody (2D6) specific for a late viral antigen followed by staining with an FITC-conjugated anti-Mouse IgG. The Figure shows scatter plot analysis of mock (left) and HHV-6-infected (right) HSB-2 cells on day 8 post-infection. Note enrichment of the infected cells in G2/M phase relative to the mock-infected cells. The vertical line indicates cut-off point for expression of the late viral protein.

Figure 5. Analysis of cell cycle progression in HHV-6-infected HSB-2 cells. The cells were stained with PKH67, aliquoted in two groups, which were either mock or HHV-6 infected. The fluorescence of the stained cells was examined on day 0, 4 and 8 post-infection by flow cytometry. The figure shows cell numbers on Y-axis, and fluorescence in arbitrary units on X-axis. Note less dilution of the stain (more fluorescence and hence less proliferation) in the virus-infected cells than in the mock-infected cells.

Figure 6. Occurrence of apoptosis in HHV-6-infected cells. The cells were stained with FITC-conjugated Annexin V and PI, and examined by flowcytometry. The Figure shows the staining of the cells on day 10 postinfection. Note enhanced staining of the virus-infected, but not of the mock-infected cells, for the two markers.

Figure 7: Expression of phosphorylated H3 in HHV-6-infected cells. The virus-infected and mock-infected cells (day 8) were incubated for 24 hours in the presence of vehicle (DMSO) or Nocodazole. After the incubation, the cells were stained intracellularly with FITC-conjugated monoclonal antibody specific for H3 phosphorylated at Serine 10. The stained cells were examined for H3 expression as well as for DNA content (after staining with PI) by flow cytometry. The panels in the upper row show cell cycle analysis based upon DNA contents. The panels in the middle row show expression of phosphorylated H3. The panels in the lower row show DNA content and expression of the phosphorylated H3 as a scatter plot.

Figure 8: Expression of cell cycle-regulatory proteins in HHV-6-infected cells on Western blots. The expression of some cell cycle-related proteins (Cyclin A, Cyclin B, cdc2 (total and phosphorylated at Tyr 15), pRb (total and phosphorylated at Serine 811 and Serine 795), p53 (total and phosphorylated at Serine 15), and p21 are shown in A and B panels. The expression of β-actin was determined as a control. Panel C shows effect of the proteasome inhibitor MG 132 on the expression of p21 in the mock- and the virus-infected cells on day 8 post-infection. The cells were incubated in the culture medium containing 2 μM MG132 for 12 hours and lysed for Western blots. The letters M and I in the Panel B indicate mock- and HHV-6 infection, respectively.

Figure 9. DNA synthesis decreases in the HHV-6-infected cells but cells with more DNA accumulate. HHV-6-infected and mock-infected cells were incubated with BrdU for 6 hours and stained with an FITC-conjugated anti-BrdU antibody. After washing, the cells were stained with PI and analysed for their DNA contents and incorporated BrdU. The Figure shows a typical scatter plot analysis of mock (left) and HHV-6-infected (right) HSB-2 cells on day 8 post-infection. Note decreased overall BrdU incorporation in the virus-infected cells, and accumulation of cells with more than G2/M DNA.

Figure 10: Determination of ploidy in HHV-6-infected cells. The virus-infected and mock-infected cells (day 8) were processed on slides for FISH using a FITC-conjugated pRb-specific probe. After counterstaining cells with DAPI, the slides were examined under a fluorescent microscope for determining the FISH spots. The figure shows typical photomicrographs of nuclei with different cell cycle phases and ploidies.

Figure 11: Co-immunoprecipitation of cdc2 and p41 in HHV-6-infected cells. A. P41 was immunoprecipitated from the HHV-6-infected and mock-infected HSB-2 cells on

day 4 post-infection. The immunoprecipitates were run on 10% SDS-PAGE, and blotted with anti-P41 or anti-cdc2 antibodies. Note co-immunoprecipitation of cdc2 with P41. B. Same as in A, except that here cdc2 was immunoprecipitated. Note co-immunoprecipitation of P41 with cdc2. Some background is seen with anti-P41 antibodies in the Mock-infected HSB-2 cells.

Figure 12: Cyclin B localizes to nuclei in HHV-6 infected cells. (A) HHV-6 infected and mock-infected cells were stained intracellularly with PE-conjugated anti-cyclin B monoclonal antibody and examined by flow cytometry. Note decreased expression of the cyclin in the virus-infected cells. (B) The cells were also stained with DAPI and examined under a confocal microscope. Note predominantly intranuclear localization of cyclin B in HHV-6-infected cells.

Figure 13: Expression of cytoskeletal proteins in HHV-6-infected cells. The virus and mock-infected cells were stained intracellularly with an FITC-conjugated anti-α-tubulin, anti-γ-tubulin antibodies, Phalloidin, DAPI, PHK-67 or virus-specific antibodies antibody. The cells were examined under a confocal microscope. **A.** The cells (day 8 post-infection) are stained intracellularly with an FITC-conjugated anti-human α-tubulin and DAPI. Note increased cell size and increased perinuclear expression of α-tubulin in the virus-infected cells. Nocodazole treated cells were used as positive controls. **B.** The cells are stained with DAPI and Phalloidin. Increased expression of F-actin and its nuclear infiltration is clearly seen in the virus-infected cells. **C.** The cells are stained with 2D6 (recognizing a late viral antigen) followed by FITC-conjugated anti-mouse IgG, red Phalloidin and DAPI (blue). Note increased expression of polymerized actin (red), and lobed nuclei (blue) in the virus-infected (2D6-positive; green) cells. **D.** The virus-infected and mock-infected (day 5 post-infection) were stained intracellularly with 9A5 (an early viral antigen) followed by staining with FITC-conjugated anti-mouse IgG, Phalloidin and DAPI. Note increased expression of polymerized actin (especially between nuclear lobes) in the virus-infected cells. The arrow indicates an infected cell. **E**. The cells (day 8 postinfection were stained for PKH-67 and PI. Note increased cytoplasm and enlarged nucleus with

segments in a virus-infected cell. **F.** The cells (day 8 postinfection) were stained intracellularly with FITC-conjugated anti-γ-tubulin, DAPI and Phalloidin. Note a bridge of F-actin and γ-tubulin between the dividing cells. The cells appear to be undergoing cytokinesis. **G.** The cells (day 8 and 12 post-infection with and without cytochalasin B) are stained intracellularly with an FITC-conjugated anti-human α-tubulin and DAPI. Note appearance of the virus-infected cells similar to the cytochalasin B-treated cells. An enlarged virus-infected cell indicated by an arrow is in the process of nuclear lobation.

Figure 14: The expression of securin decreases in HHV-6-infected cells. The cells (day 8 postinfection) were stained intracellularly with FITC-conjugated anti-human securin, DAPI and Phalloidin (F-actin), washed and examined under a confocal microscope. Note cytoplasmic expression of the protein in mock-infected cells and its marked decrease in the virus-infected cells.

Figures

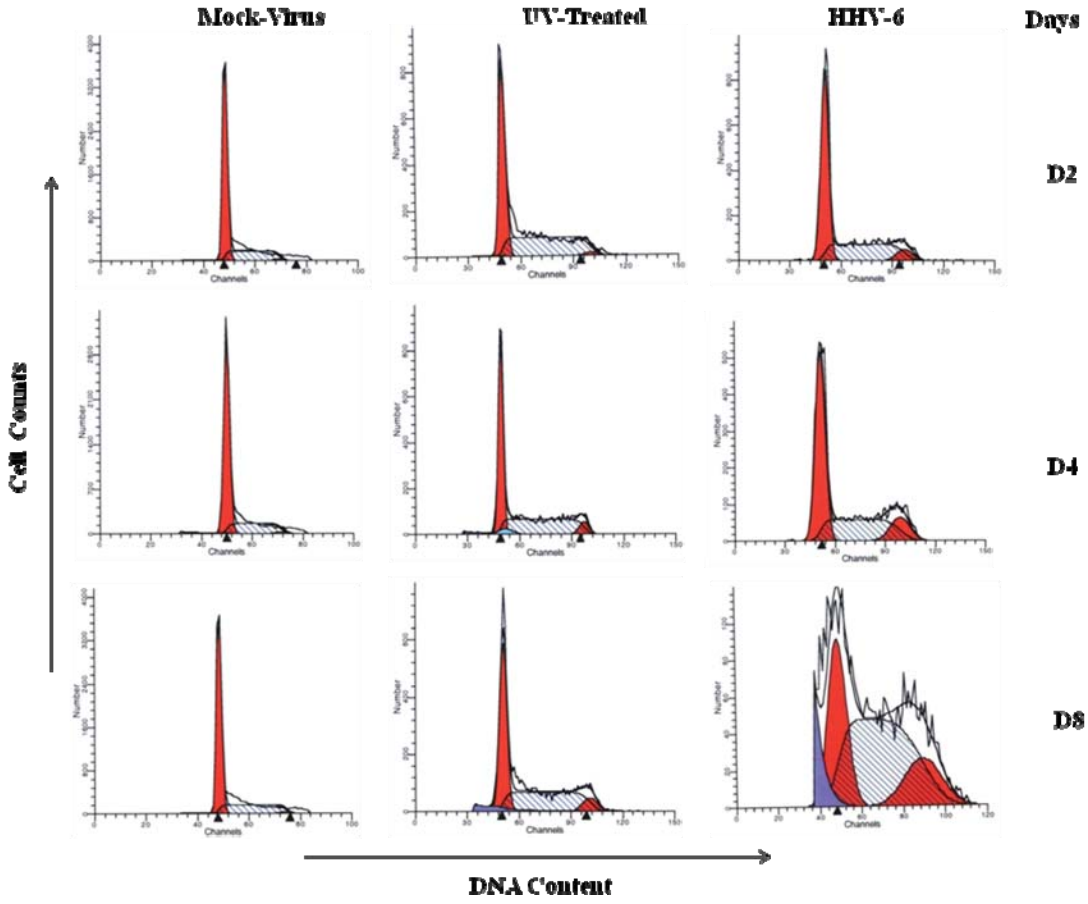
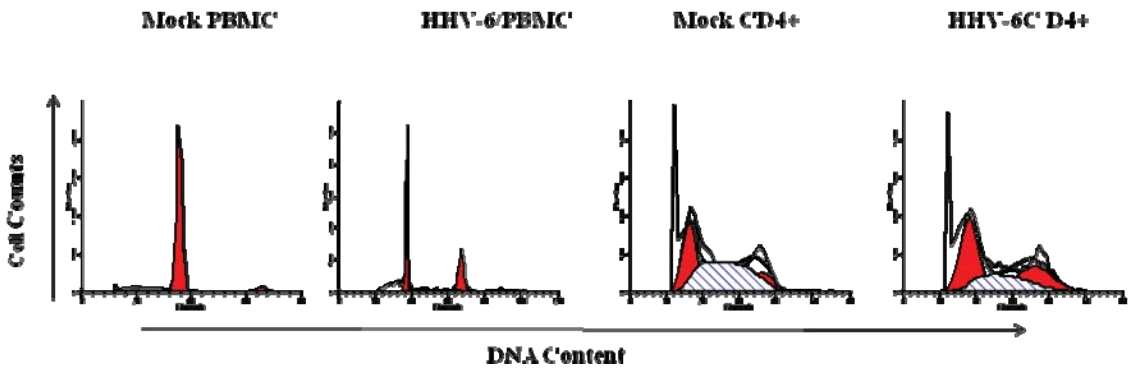


Figure 1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HHV-6/PBMC
Mock CD4+
HHV-6C D4+
Cell Counts
DNA Content

Figure 2

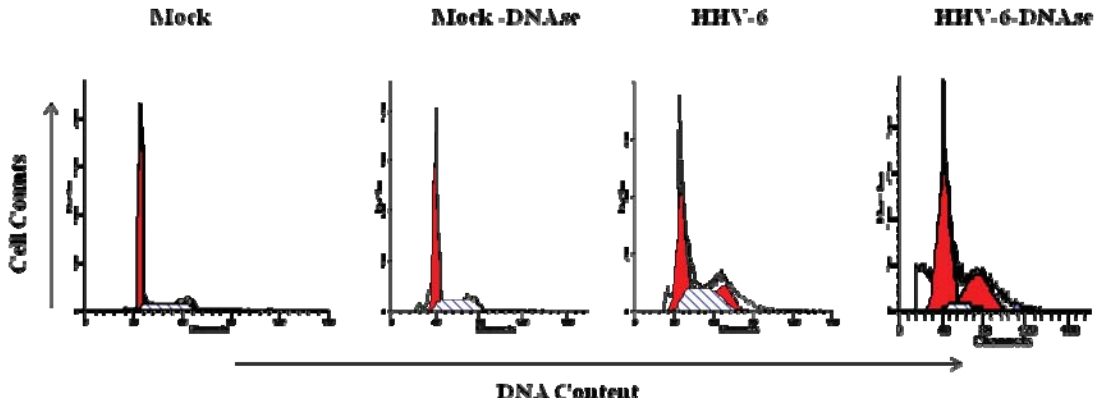
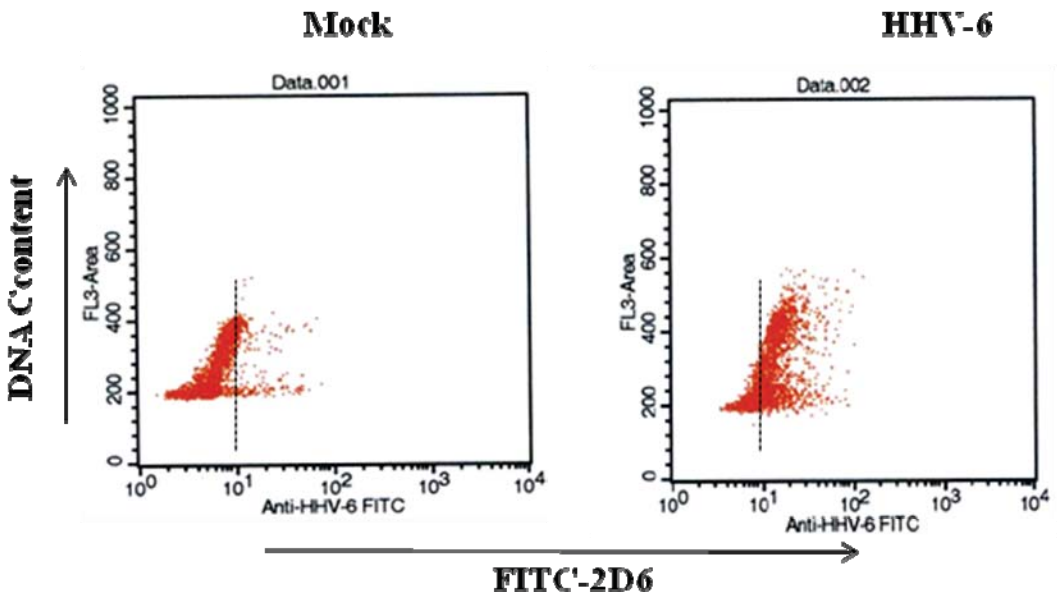


Figure 3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Data.002
DNA Content
FL3-Area
Anti-HHV-6 FITC
FITC-2D6

Figure 4

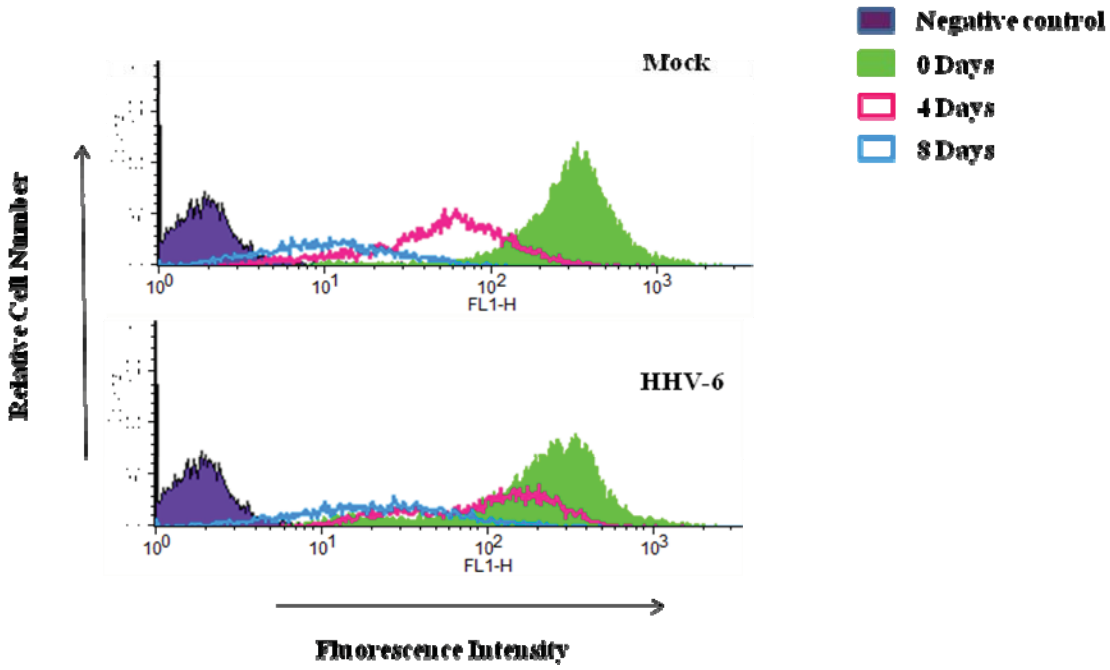

Negative control
0 Days
4 Days
8 Days
Mock
HHV-6
Relative Cell Number
FL1-H
Fluorescence Intensity

Figure 5

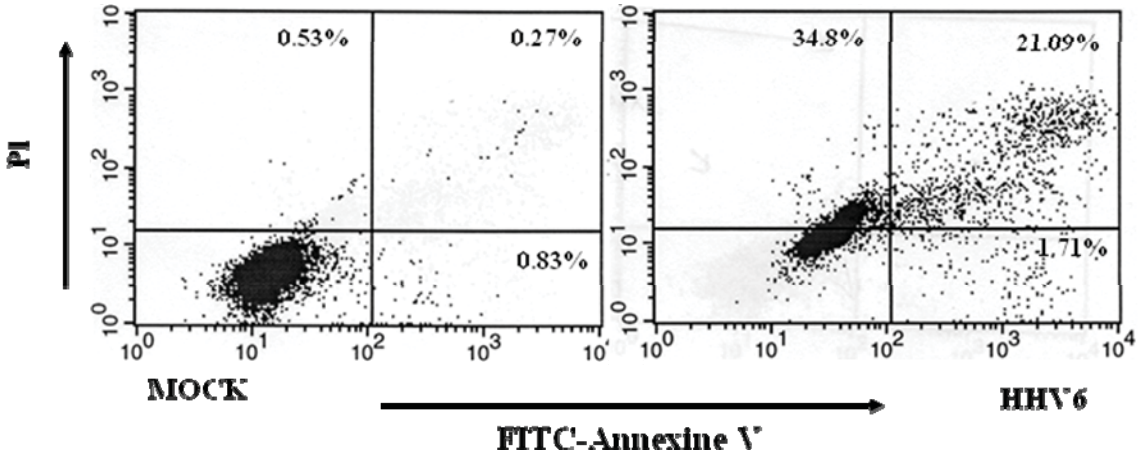
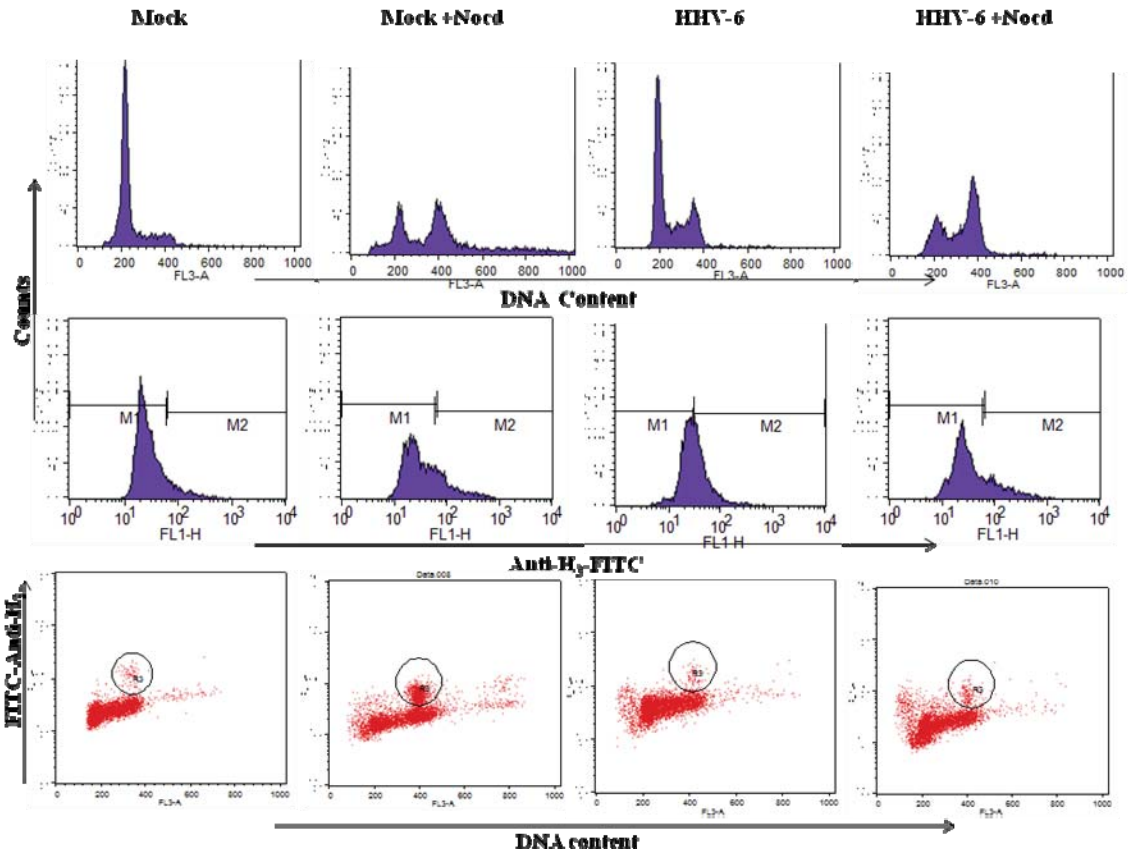


Figure 6


Mock
Mock +Nocd
HHV-6
HHV-6 +Nocd
Counts
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DNA Content
M1
M2
FL1-H
Anti-Hγ-FITC
FITC-Anti-Hγ
DNA content

Figure 7

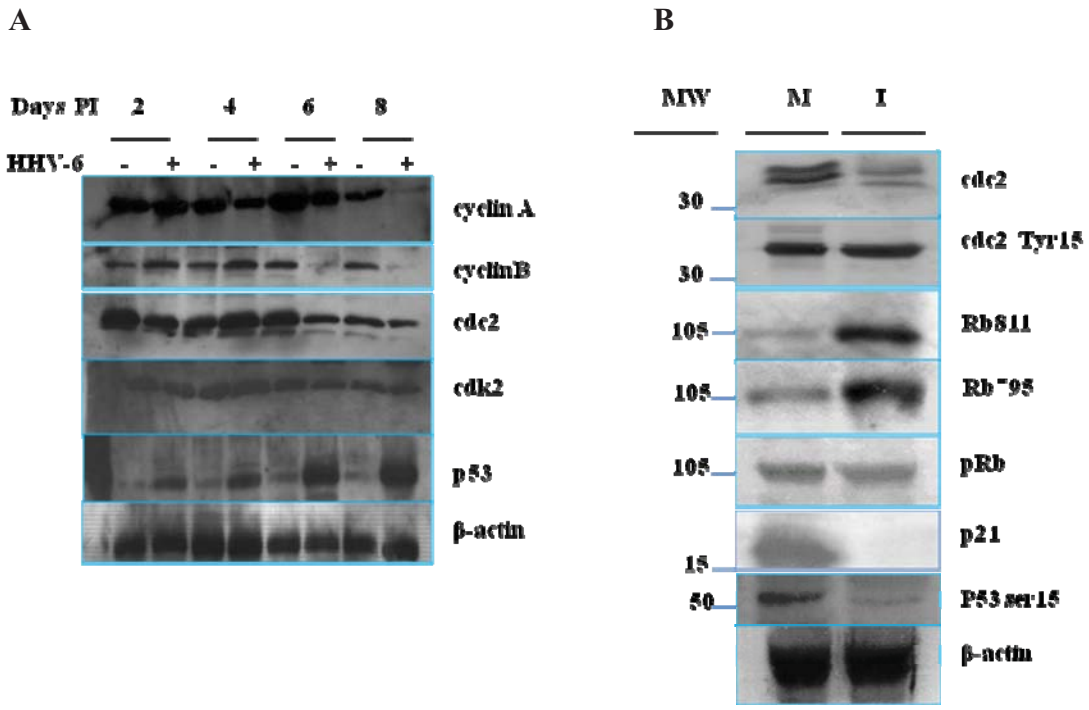
A
Days PI 2 4 6 8
HHV-6 - + - + - + - +
cyclin A
cyclinB
cdc2
cdk2
p53
β-actin
B
MW M I
30
30
105
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Figure 8

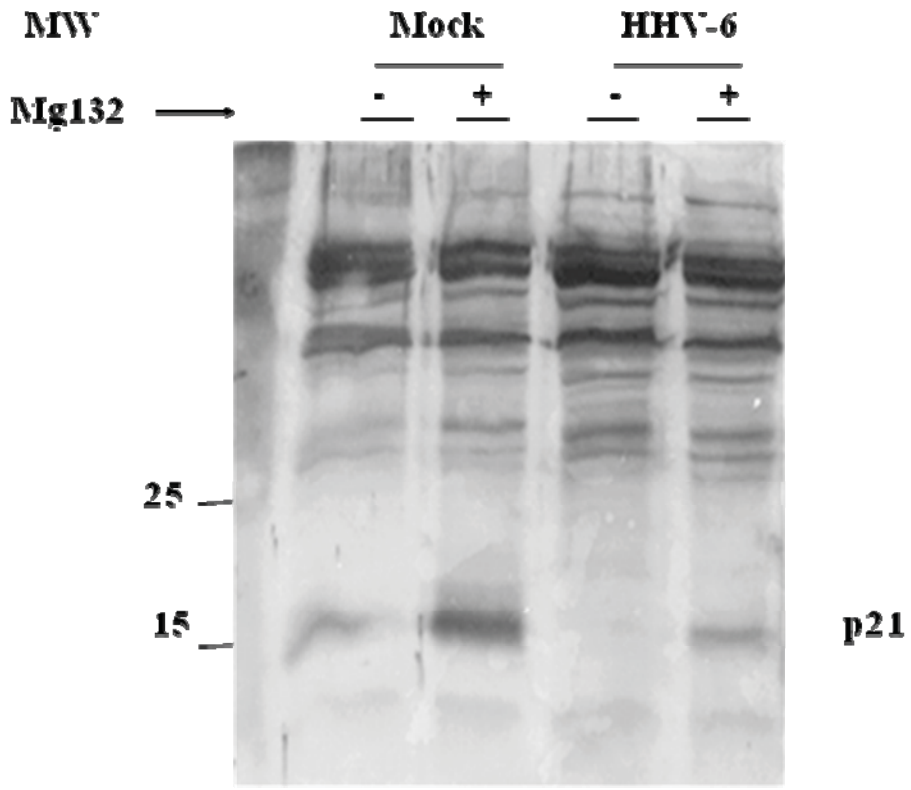

Figure 8C

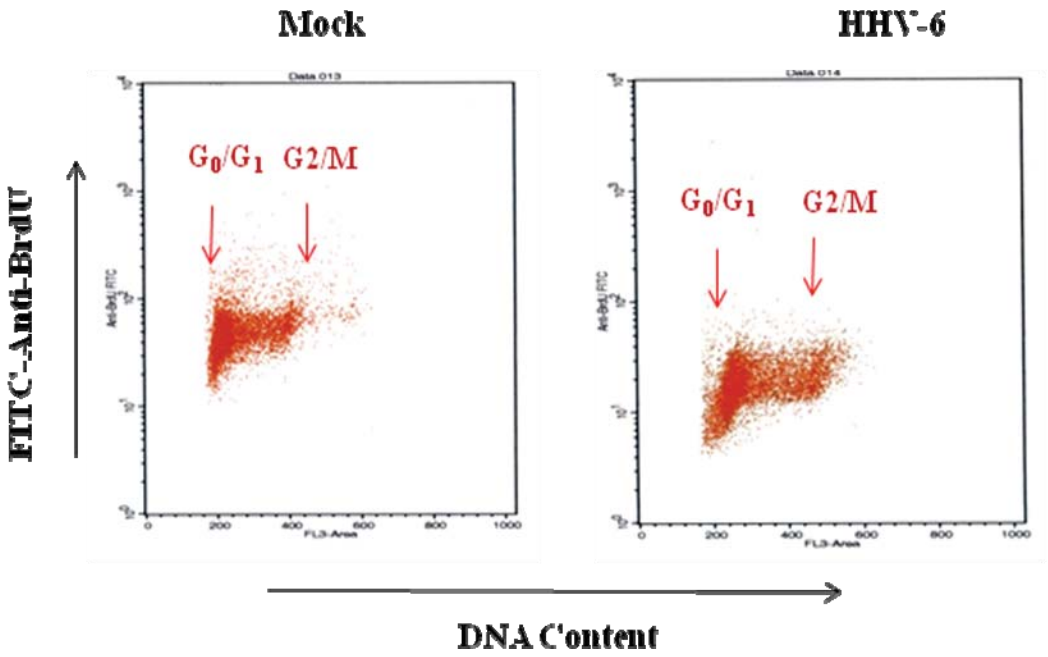

Figure 9

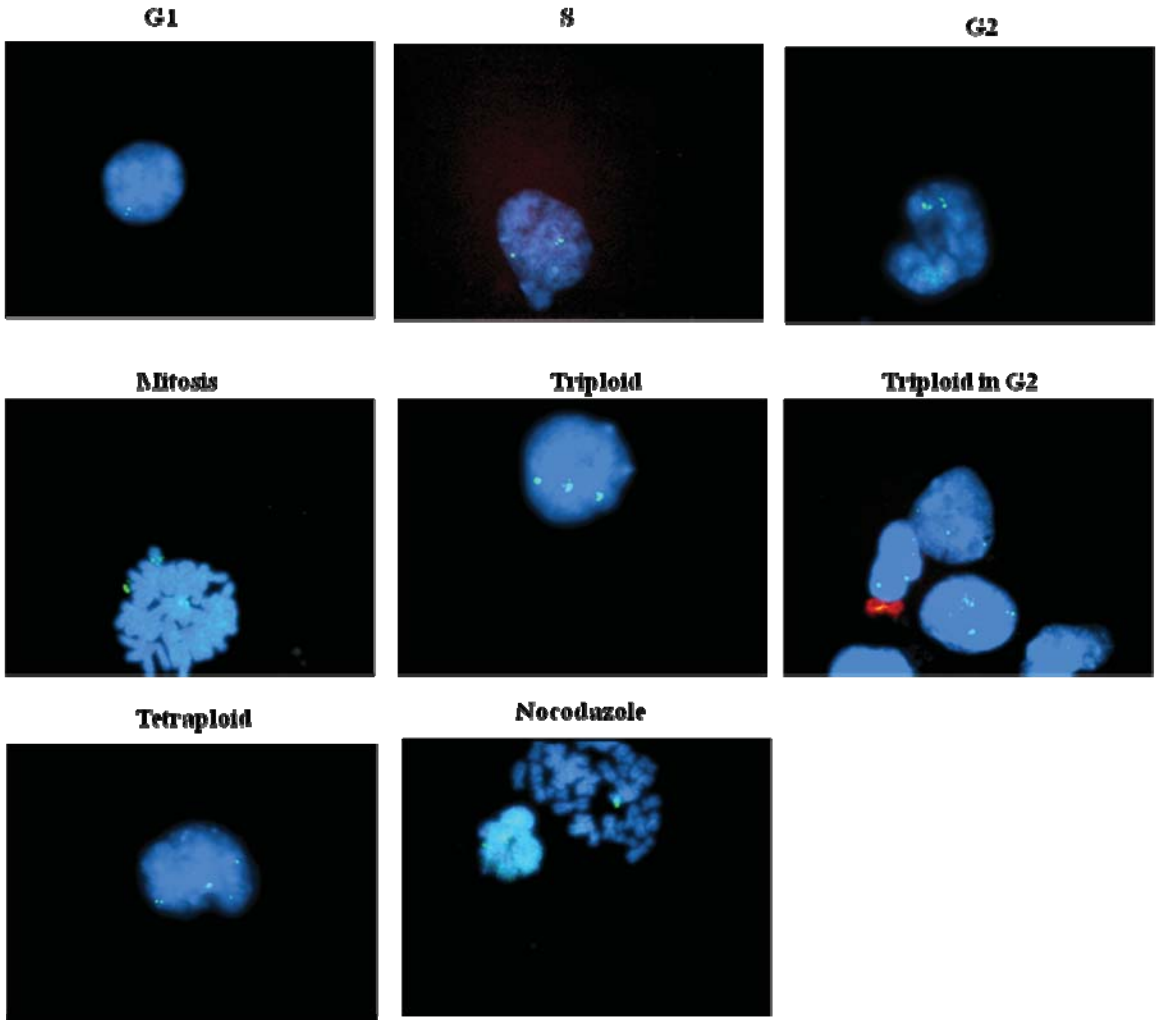
G1
S
G2
Mitosis
Triploid
Triploid in G2
Tetraploid
Nocodazole

Figure 10

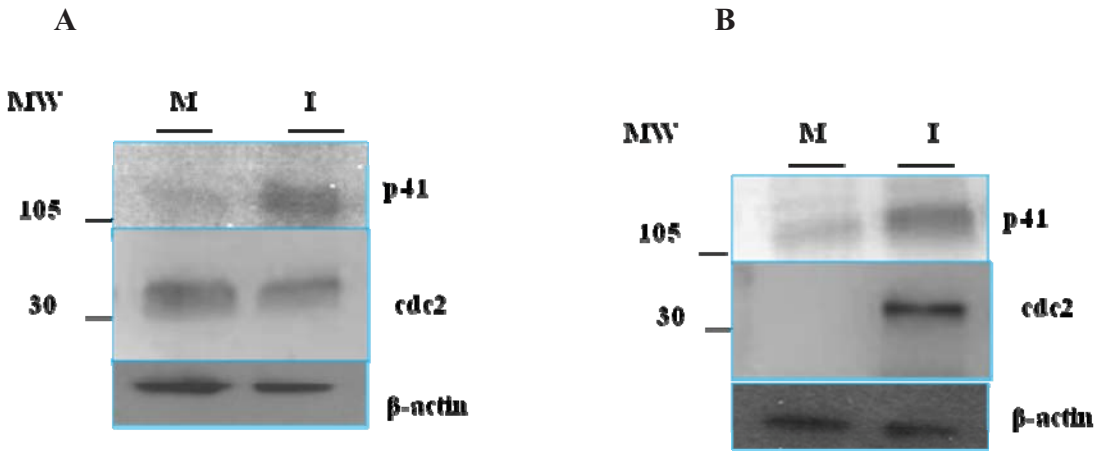

Figure 11

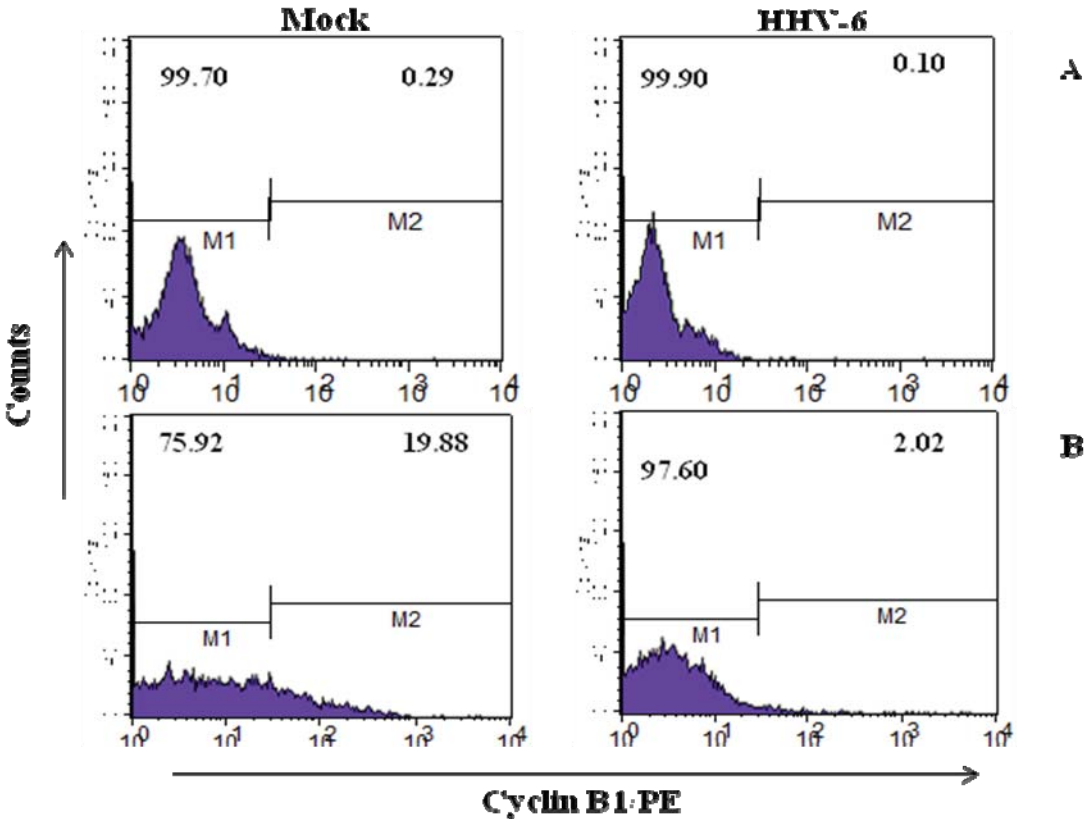
Mock
HHV-6
99.70
0.29
99.90
0.10
A
M1
M2
M1
M2
Counts
75.92
19.88
97.60
2.02
B
M1
M2
M1
M2
Cyclin B1-PE

Figure 12A

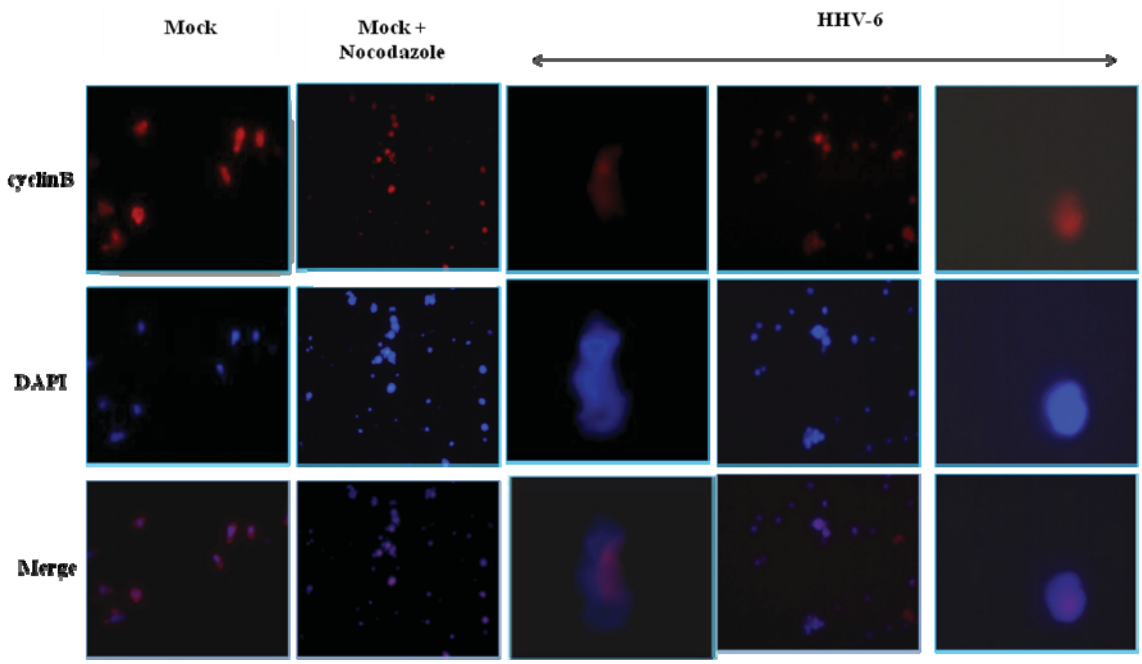

Figure 12B

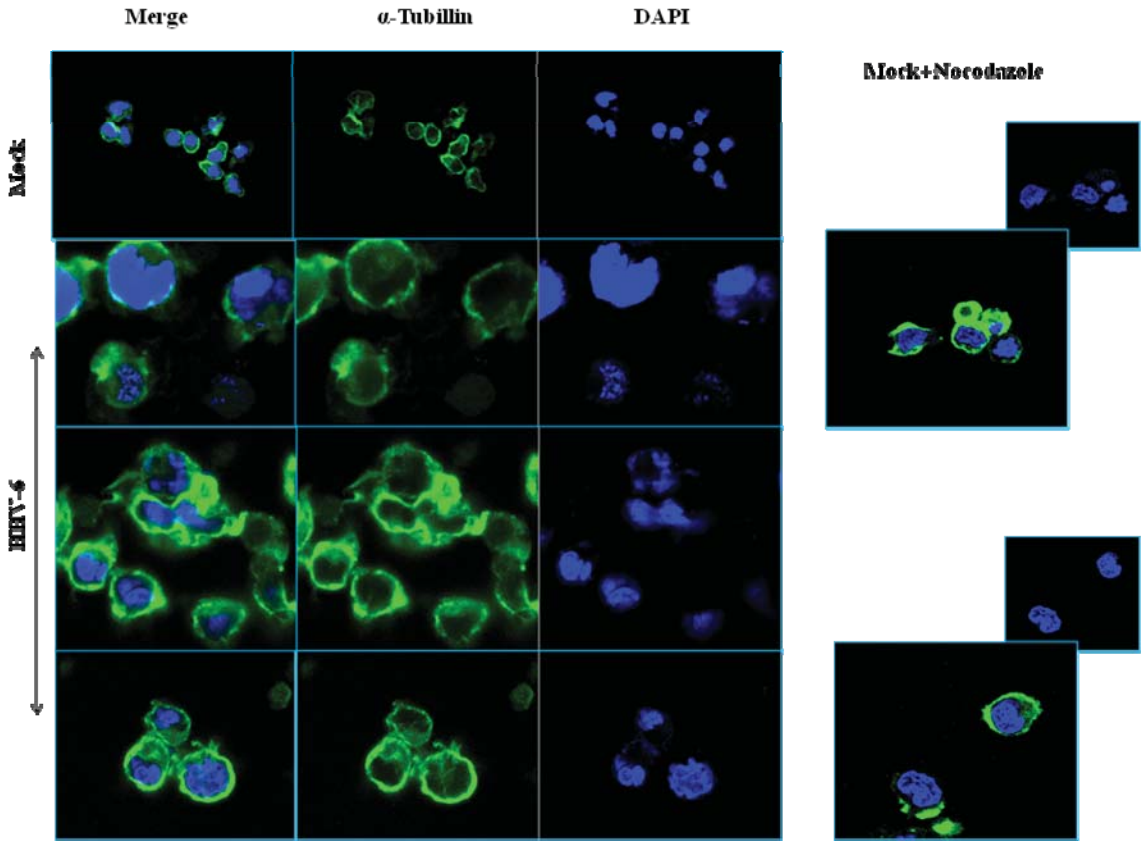

Figure 13A

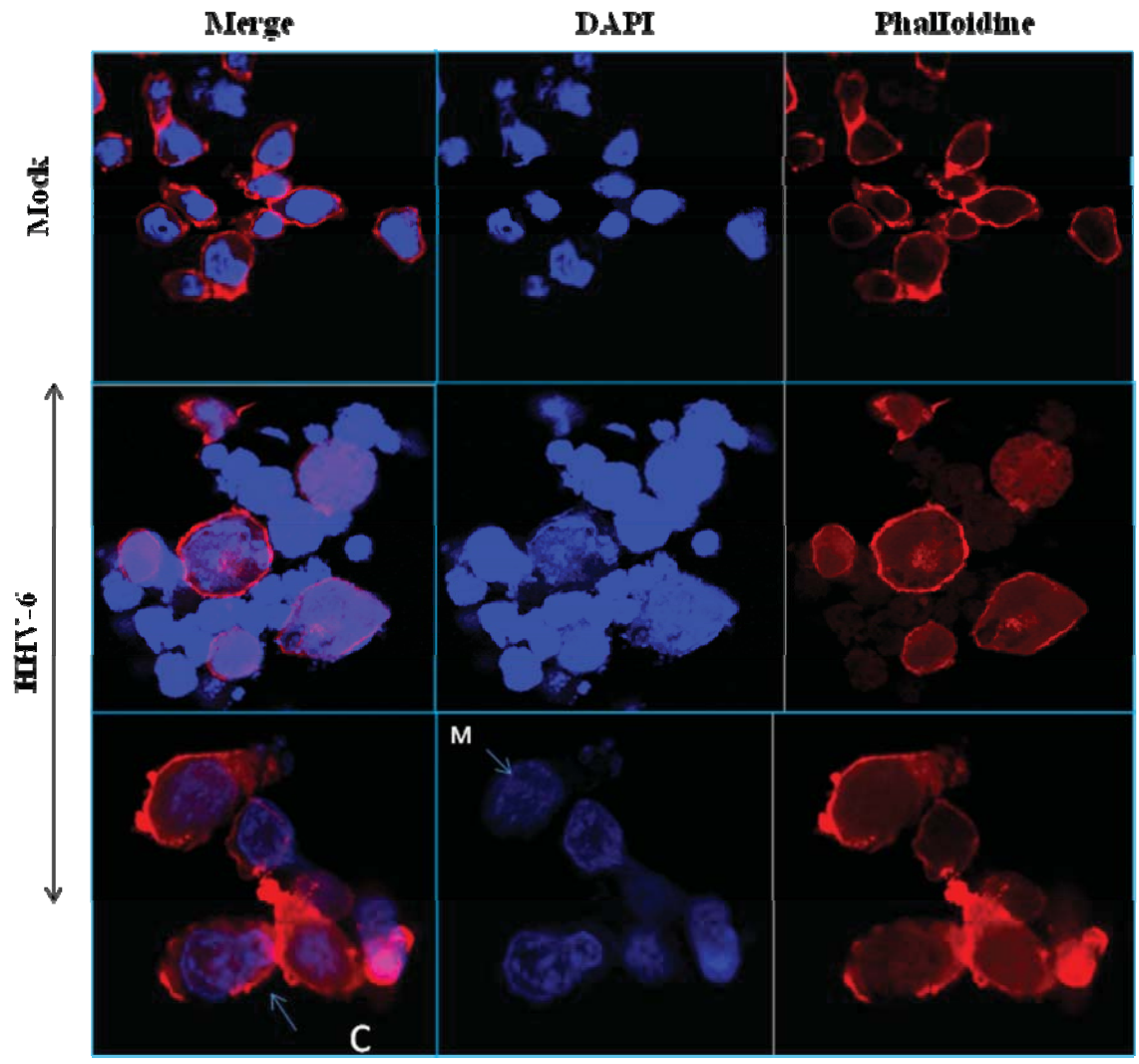

Figure 13B

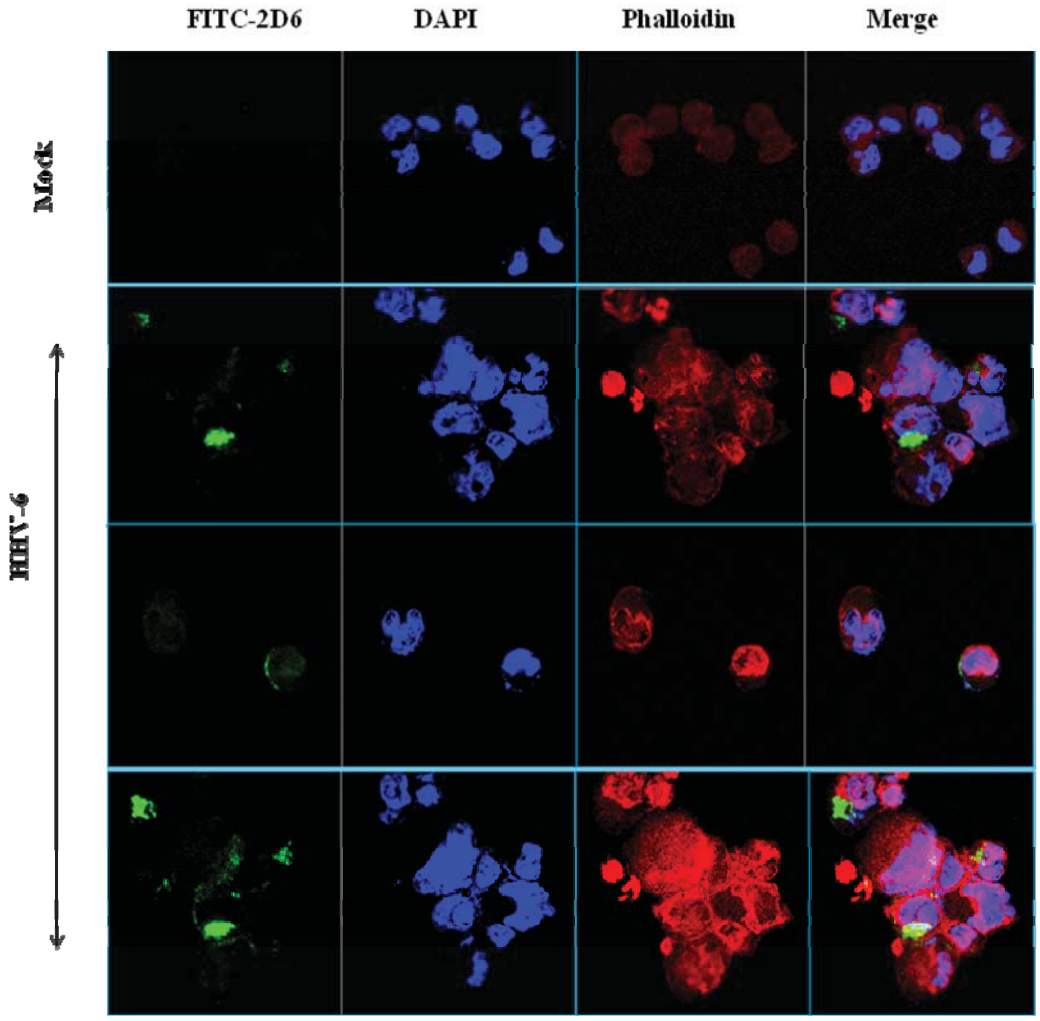

Figure 13C

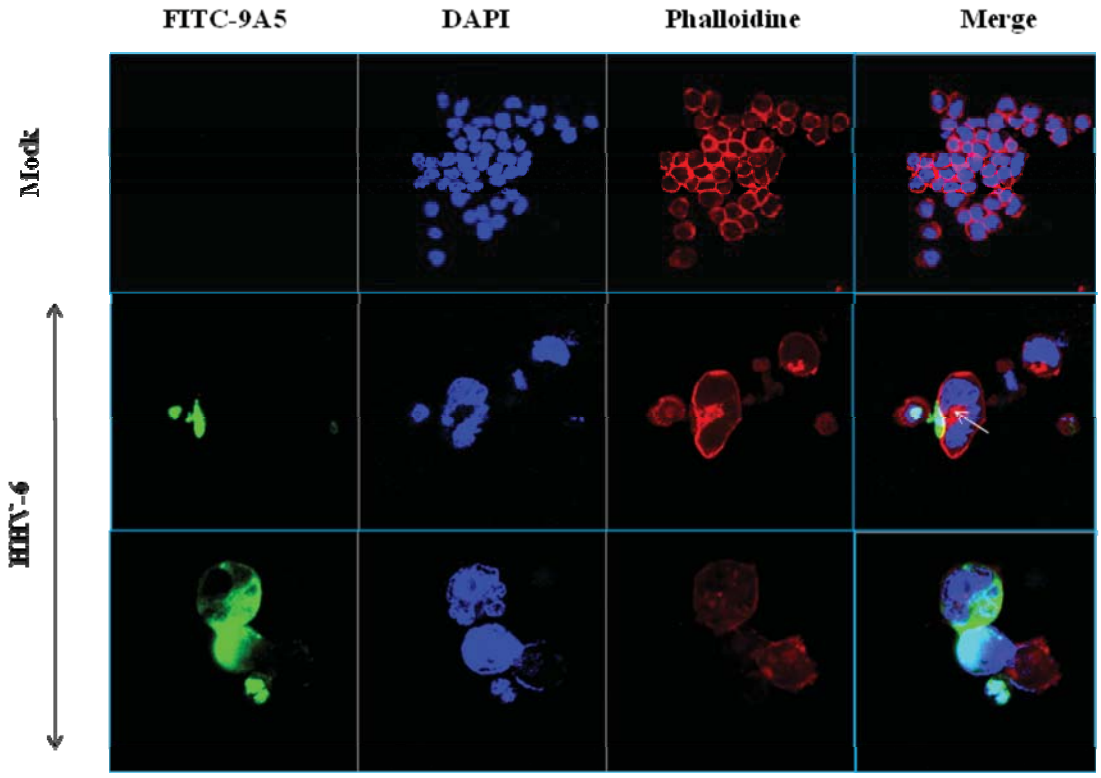

Figure 13D

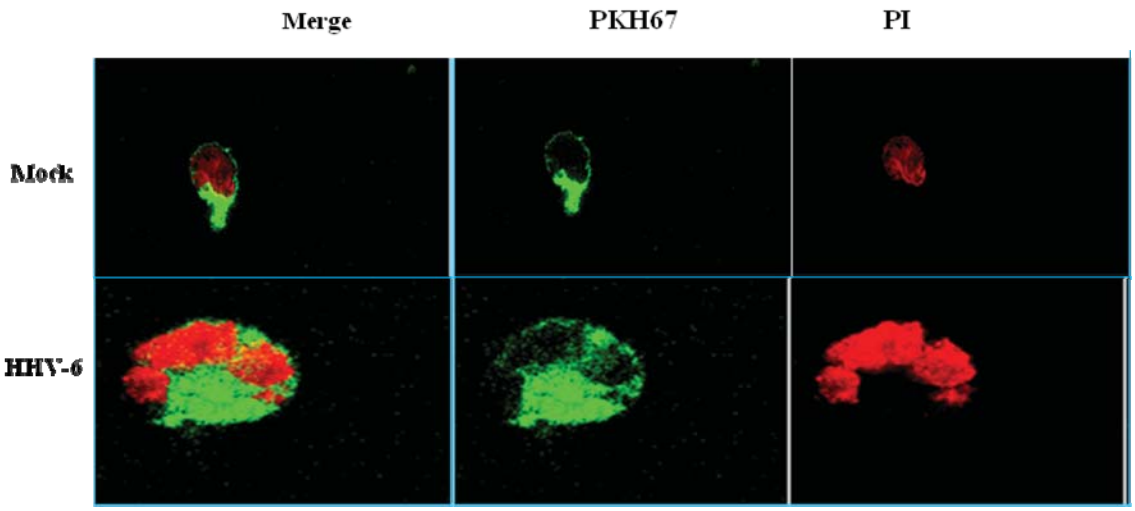

Figure 13E

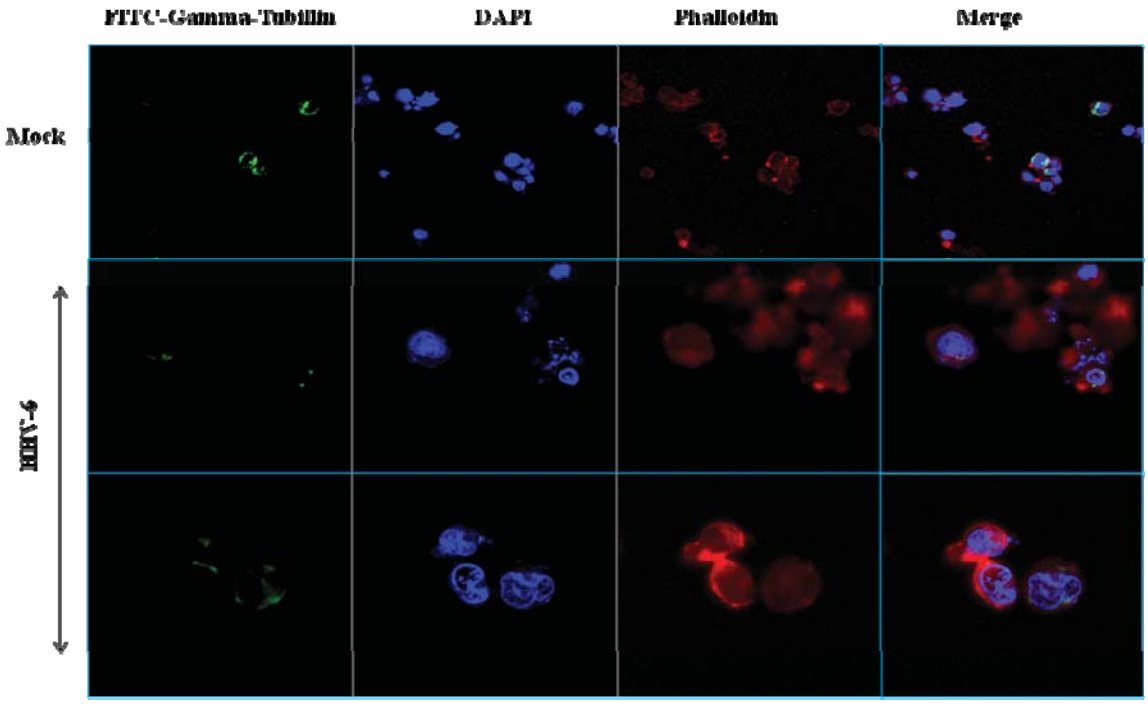

Figure 13F

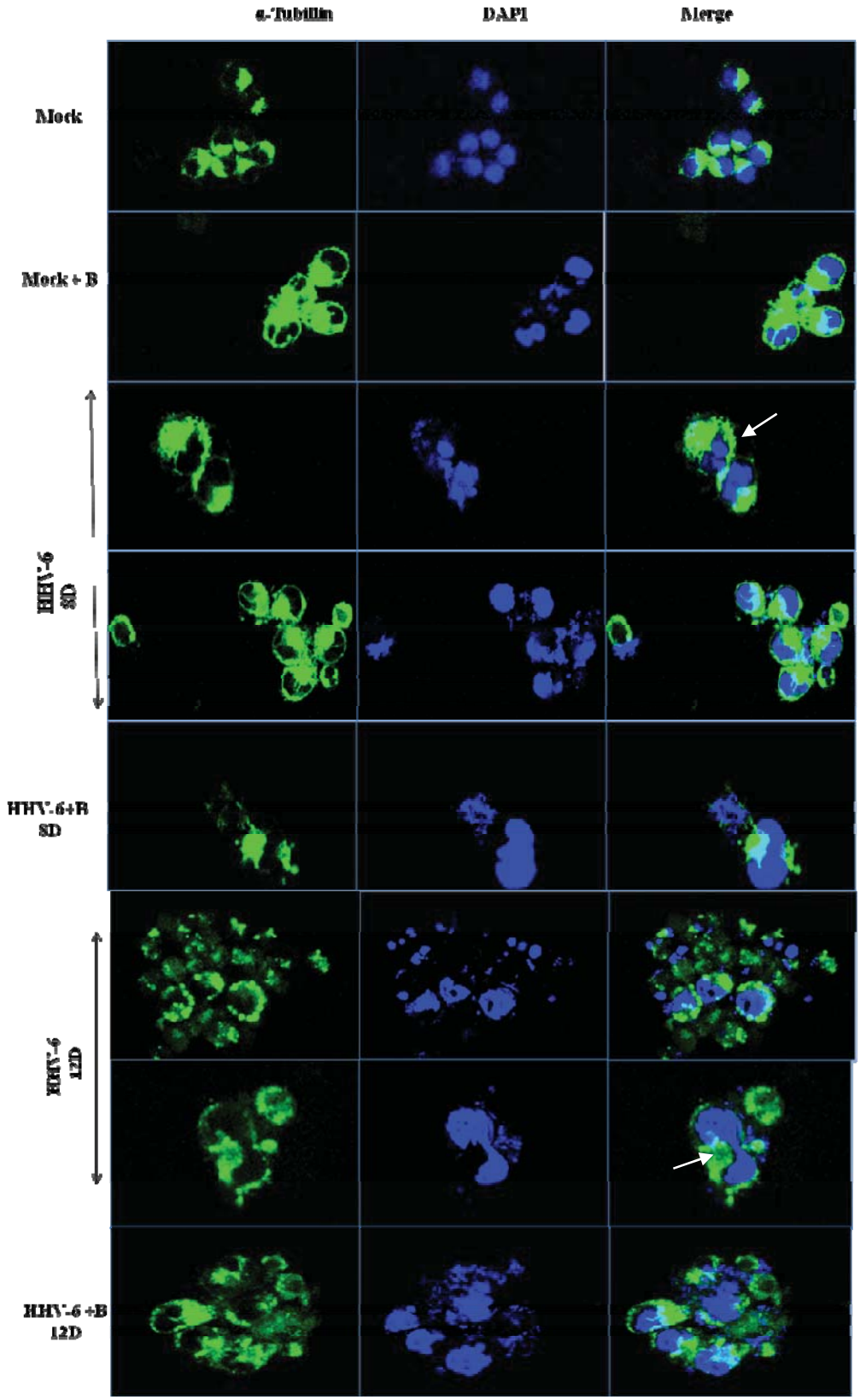
α-Tubillin
DAPI
Merge
Mock
Mock + B
HHV-6
8D
HHV-6+B
8D
HHV-6
12D
HHV-6 +B
12D

Figure 13G

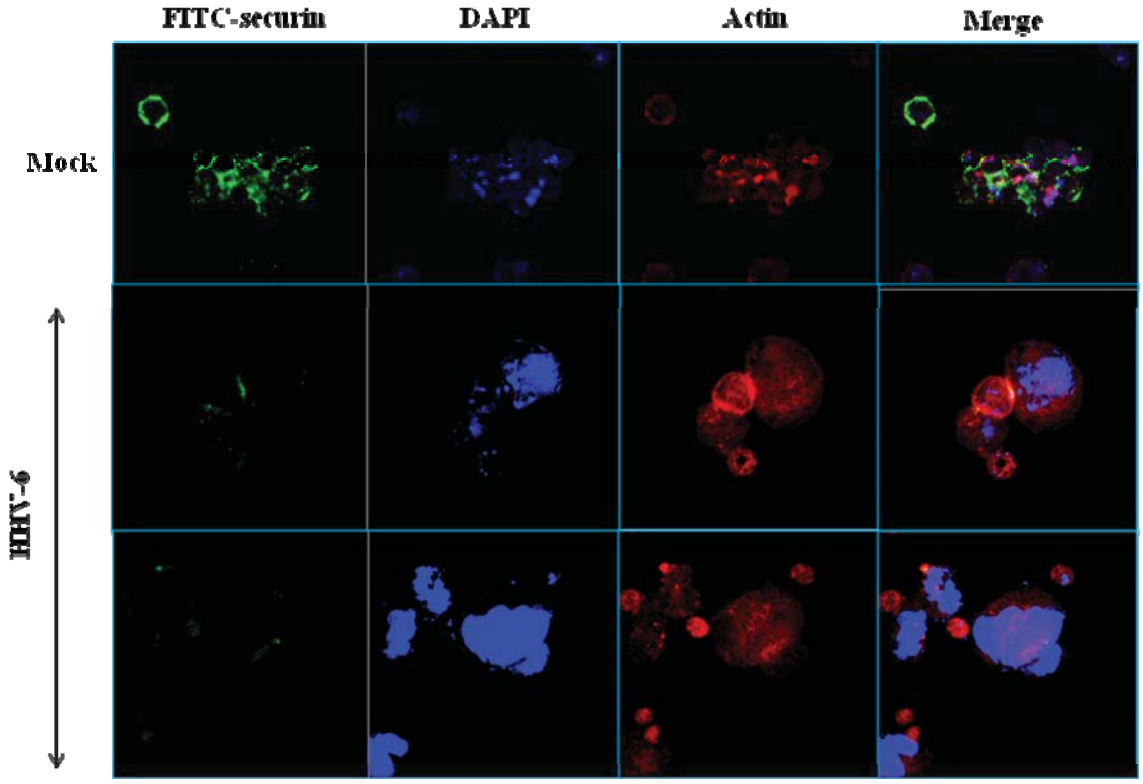

Figure 14

Résultats 2: HHV-6 modifies autophagic response of the host cells and prevents fusion between autophagic vacuoles and lysosomes in human T cells

Article 3: Olfa Debbeche, Suzanne Samarani, James Dixon, Alberto Severini and Ali Ahmad

Résumé: L'herpèsvirus humain (HHV) -6 est un agent pathogène ubiquitaire qui infecte les humains lors de la petite enfance. Ce virus provoque la roséole chez les enfants, et il a été associé à une variété de syndromes lymphoprolifératifs et des maladies chroniques chez l'homme, en particulier dans des conditions d'immunosuppression. En général, les cellules hôtes répondent aux infections virales par l'induction d'une réponse autophagique alors que les virus ont tendance à modifier cette réponse de l'hôte et de l'exploiter à leur avantage. Nous montrons ici pour la première fois que l'infection virale induit la réponse autophagique dans une lignée cellulaire établie T humaine; HSB2. Par conséquent, des vacuoles autophagiques apparaissent dans les cellules infectées par ce virus. Toutefois, la fusion entre les vacuoles et des lysosomes autophagiques ne se produit pas dans les cellules infectées par HHV-6. L'inhibition de la réponse avec le 3-méthyladenine, augmente la réplication virale dans ces cellules infectées, alors que sa promotion par la rapamycine la diminue. Ces résultats fournissent de nouvelles informations importantes sur le HHV-6 et la réponse autophagique de l'hôte.

HHV-6 modifies autophagic response of the host cells and prevents fusion between autophagic vacuoles and lysosomes in human T cells

Olfa Debbeche[1], Suzanne Samarani[1], James Dixon[1], Alberto Severini[2] and Ali Ahmad[1]

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Running Title: HHV-6 prevents fusion between autophagic vacuoles and lysosomes

Abstract

The Human herpesvirus (HHV)-6 is a ubiquitous pathogen that infects humans in early childhood. The virus causes roseola in children, and has been associated with a variety of lymphoproliferative disorders and chronic diseases in humans, especially under conditions of immunosuppression. In general, the host cells respond to viral infections by inducing autophagic response and the viruses tend to modify this host response and exploit it to their own advantage. We show here for the first time that the viral infection induces autophagic response in an established human T cell line HSB2. Consequently, autophagic vacuoles appear in the virus-infected cells. However, the fusion between autophagic vacuoles and lysosomes does not occur in these cells. Inhibition of the response with 3-Methyladenine increases, and its promotion with Rapamycin decreases, viral replication in these cells. These results provide important novel insights on HHV-6 and host's autophagic response.

Key words: Autophagy, HHV-6, HSB-2, LAMP-1, LC-3

Introduction

Autophagy or "self-eating" represents a physiological cellular process in which cells degrade aged and damaged cellular organelles, misfolded proteins, large bits of the cytoplasm, long-lived cellular proteins as well as their abnormal aggregates through lysosomes; reviewed in (1). Autophagy involves a cascade of processes in which the to-be-degraded cytoplasmic constituents are sequestered by *de novo* developed double-membrane cytosolic vesicles called autophagosomes or autophagic vacuole (AV). Proteins from almost twenty Autophagy-related (Atg) genes participate in the process, which has been well studied in eukaryotic cells (1, 2). During this process, a protein called LC-3 (the microtubule light chain-3 like; the product of the Atg-8 gene) is cleaved and is lipidated by conjugation with phosphatidylethanolamine. The lipidated LC-3 (called LC-3 II, whereas the uncleaved and unlipidated form is called LC-3 I) associates with the AV membranes, and tethers autophagic cargo into the vacuole (3, 4). The AV undergoes maturation and fuses with lysosomes forming autolysosomes. This leads to degradation of the autophagosome contents. Deficiency or mutations in the Atg-5, Atg8 or Atg-7 genes render cells incapable of mounting autophagy. Autophagy-deficient organisms show enhanced susceptibility to cancer (5); reviewed in (6).

The autophagic process occurs at low basal levels in eukaryotic cells and plays an important role in their metabolic homeostasis and proper janitorial functions. The degraded cellular components are recycled as a source of energy for cell metabolism. Exaggerated autophagy, however, may also kill cells. It has been shown that the autophagic process is enhanced in cells undergoing starvation. By degrading its own cytoplasmic contents during starvation, the cells try to provide themselves with nutrients, meet their energy demands and extend their life-span, albeit transiently (7). They also use this process to sequester and eliminate foreign pathogens like viruses. In this context, the process has been termed as xenophagy, and more specifically against viruses as virophagy. It is not surprising that viruses

have evolved strategies to evade this process and rather use it to their advantage (8);reviewed in (9).

HHV-6 is a ubiquitously occurring human pathogen. It is a lymphotropic β-herpesvirus that usually infects humans in early childhood. In most populations of the world, more than 95% individuals become seropositive by 2 years of age (10, 11), reviewed in (12, 13). Primary infections with the virus usually cause a mild febrile illness called roseola or exanthum subitum (14). The condition is usually self limiting. Rarely, the infection may cause encephalitis and other central nervous system complications, and cause death (15). In adults, the primary infections result in mononucleosis-like illness (16, 17). The infected persons always become life-long carriers of the virus. They are never able to get rid of the virus. They intermittently shed the virus in their saliva. Healthy immunocompetent persons are able to control the viral infection, which often becomes reactivated under conditions of immunosuppression. Chronic HHV-6 infections have been associated with many diseases and syndromes including, multiple sclerosis, chronic fatigue syndrome, the "gloves and socks" syndrome, Rosai-Dorfman, Burkitt's lymphoma, non-Hodgkin's lymphomas, and acute lymphoblastic leukemia (ALL), etc (18-20), reviewed in (12, 21). CD4+ T cells are main targets of the virus, and a human T cell line HSB-2 is the most permissive to the virus. The virus can also infect other cell types including NK cells, CD8+ T cells, dendritic cells and monocyte-macrophages (22-24). The virus inhibits proliferation of human T cells and their production of cytokines like IL-2 (25, 26). We, and others, have shown that the infection delays cell cycle progression in the G2/M phase (27-29). It is noteworthy that autophagy plays an important role in cell cycle progression. The occurrence of autophagy is regulated as cells progress through the cell cycle. The G2/M phase, in which the virus-infected cells are delayed, is not permissive to autophagy (30, 31). This suggests that HHV-6 may be able to regulate autophagic response of the infected cells. To the best of our knowledge, so far no study has been conducted to determine the impact of the virus on the autophagic response of the host cells. This may be of interest, as other Herpesviruses have been shown to modulate this host response to their advantage,

and the host cell's autophagic responses may be targeted for the development of antiviral drugs (32, 33); reviewed in (34). Therefore, we undertook this study and determined the impact of the virus on autophagy in human cells.

Materials and Methods:

Virus stock:

An A strain of HHV-6 (GS) was used in this study. The virus was grown in HSB-2, an immature human T cell line, as described earlier (25). For this purpose, about 5 x 10^6 cells were infected with the virus by incubation at 37° C for one hour, washed with sterile PBS and incubated at 37° C in a humidified 5% CO_2 atmosphere. The cells showed cytopathic effects on day 10-12 post-infection. At this point, the culture supernatants were collected, centrifuged at 1000g for 15 minutes, and passed through 45 μ filters to remove cellular debris. The infected cell pellets were re-suspended in 1-2 ml culture medium, frozen and thawed three times to release intracellular virus. Their supernatants were treated like the culture supernatants, and were added to the infected cell culture supernatants. The supernatant served as the viral stock. It was titrated for TCID50, aliquoted and stored at –80° C. The supernatants collected from uninfected HSB-2 cells were treated as for the supernatants from the virus-infected cells and were used for mock infections. The viral stocks thus prepared had a titre of 5-9x 10^5 $TCID_{50}$ per ml.

Cell culture and viral infections:

For these studies, we used HSB-2 cell line, which was established from an acute T cell leukemia patient (ATCC, Bethesda, MD). The cells were cultured in the medium RPMI-1640 (Gibco/BRL, Burlington, Canada) supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 2.5 mM L-glutamine. To the culture medium, antibiotics (100 units per ml of penicillin from Novapharm, Toronto, Canada; 2 μg per ml of gentamycin from Schering, Pointe Claire, Canada and 2.5 μg per ml of fungizone from Squibb, Montreal, Canada) were also added. The cell cultures were incubated in the culture medium at 37° C in 5% CO_2-containig humidified incubators. The medium was changed when it became yellowish. For each infection, about five million cells were infected with HHV-6 at a moi of 0.2 by incubating them with 2 ml of the viral preparation at 37° C for one hour. The cells were gently and intermittently shaken during this incubation, washed, re-suspended in the culture medium and incubated at 37° C. The infection

protocol usually led to 95% infection rate when examined by an indirect immunofluorescence assay with a virus-specific monoclonal antibody 2D6, as described earlier (35).

Determining autophagy

The occurrence of autophagy was determined by aggregation and accumulation of LC-3 in cells. For this purpose, the cells were fixed and permeabilized using a kit Cytofix/Cytoperm (BD Biosciences; 554722). The cells were incubated on ice for 30 minutes with a mouse monoclonal anti-human LC-3 antibody (clone 4E12; Code M152-3; MBL, Japan). After washing with cold PBS, the cells were incubated with FITC-conjugated anti-mouse IgG antibodies (Biolegend; 405305) for 45 minutes. After washings, the cells were examined under a confocal microscope. The cells undergoing autophagy showed a characteristic punctuate distribution of LC-3. Normal cells, not undergoing autophagy, show diffuse staining in the cytoplasm. As a positive control for autophagy, we incubated cells with Rapamycin (1 μg per ml; Cell Signalling; 9904), which is known to inhibit mTOR and induce autophagy in eukaryotic cells (36).

In some experiments, the virus-infected and mock-infected cells were treated with an inhibitor of autophagy, 1 μg per ml 3-Methyladenine (3MA; Sigma; M9281). The reagent inhibits class III PI-3K that are needed to form autophagic vacuoles (37).

Western blots:

An essential characteristic of autophagy is the cleavage and lipidation of LC-3 I into LC-3 II, which migrates more rapidly on SDS-PAGE and can be detected in Western blots (3). We detected LC-3 I and LC-3 II in the virus and mock virus-infected cells by this method. Briefly, 5 x 10^6 cells were washed with PBS and lysed in a lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 2mM DTT and boiled for 15 seconds as described earlier (38, 39). The particulate matter was removed from the lysates by centrifugation at 14000g for 30 minutes at room temperature and their protein concentrations were determined with a commercial

kit (BCA TM protein assay Kit from Pierce; catalogue # 23225). Fifty μg of the cell lysate proteins were loaded on 12% SDS-PAGE in the sample buffer as described (38, 39). The resolved proteins from the gel slabs were transferred onto polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA) using a semi-dry transfer system (Bio-Rad). To block unbound sites on the blots, they were incubated in a blocking buffer (1x casein solution, Vector Laboratories Inc) at room temperature for two hours. Thereafter, the blots were incubated with anti-LC-3 antibodies Specific protein bands were revealed on the blots by enhanced chemiluminescence by using a commercial kit (Vectastain ABC-AMP; Vector Laboratories, AK-6601).

Determination of HHV-6 replication:

HHV-6 replication was determined by using a commercial kit (CMV HHV-6,7,8 R-gene™ Quantification Kit; Argene). The kit determines viral genomes in a given sample by using real time PCR that amplifies a 116 bp-fragment from the U57 gene using gene-specific primers. DNA is extracted from specimens and quantified by real-time PCR. The results are reported as copy number of HHV-6 DNA per mL of original specimen.

Statiscal Analysis

Group means were compared using students't test and ANOVA. Differences were deemed significant at $p \leq 0.05$.

RESULTS

HHV-6 infection induces autophagy in HSB-2 cells

We examined HHV-6-infected, mock-infected and Rapamycin-treated HSB-2 cells after staining with FITC-conjugated anti-LC-3 antibody and DAPI under confocal microscope, as described in the Materials & Methods section. As shown in Figure 1, the infection induced accumulation of small aggregates of LC-3 in the cytoplasm of the virus-infected cells. These aggregates of LC-3 are known to

represent autophagic vacuoles. As a positive control, we treated these cells with Rapamycin, which is known to induce autophagy by inhibiting mTOR (36). The Rapamycin treatment for 24 hours also induced autophagic vacuoles in the cytoplasm of the treated cells. The Rapamycin-induced vacuoles, however, were bigger as compared to those seen in the virus-infected cells (Figure 1). The vacuoles were seen rarely in the mock-infected cells, and if present, were much smaller than those seen in the virus-infected or Rapamycin-treated cells (Figure 1). In order to confirm the occurrence of autophagy, we determined the expression of lipidated LC3 (LC3-II) in the virus-infected and Rapamycin treated cells. As shown in Figure 2, the virus-infected cells induced as much LC3-II as the Rapamycin-treated cells. The induction was, however, much higher in the cells that were infected with the virus and treated with the drug. These data also suggest an additive effect of the infection and of Rapamycin treatment on the magnitude of autophagy in these cells.

In separate experiments, we determined the effects of Rapamycin and 3-MA on the HHV-6-induced autophagy in HSB-2 cells. For this purpose, we counted the number of cells with autophagic vacuoles under a confocal microscope. As shown in Figure 3, the viral infection induced autophagic vacuoles in a higher %age of cells on day 8 post-infection than 24 hour treatment with Rapamycin. As expected, the treatments with 3-MA significantly ($p<0.05$) decreased the %age of cells with autophagic vacuoles in the HHV-6-infected HSB-2 cells. On the other hand, treatment with Rapamycin did not significantly enhance the %age of cells with autophagic vacuoles ($p>0.05$). The treatment, however, visibly increased size of the vacuoles (Figure 1). We also observed that autophagic vacuoles became detectable on day 3 postinfection and increased progressively in size with increase in cytopathic effects of the virus (increased cell size; Figure 4 and data not shown). These results suggest that HHV-6 induces autophagic response in the human cells, which is suppressed by 3-MA and is augmented by Rapamycin.

The infection prevents fusion between autophagosomes and lysosomes

Certain viruses (e.g., Hepatitis C Virus; HCV) induce formation of autophagic vacuoles but prevent their fusion with lysosomes. Consequently, the vacuoles remain negative for lysosomal markers (40). Therefore, we sought to determine whether the HHV-6-induced autophagic vacuoles fuse with lysosomes or not. For this purpose, we stained HHV-6-infected and mock-infected cells intracellularly with Rhodamine-conjugated anti-LC-3 antibodies as well as with FITC-conjugated anti-LAMP-1 (a lysosome-specific marker), and examined colocalization of the two markers via confocal microscopy. We used Rapamycin-treated cells as a positive control to ensure that our cells had the intrinsic ability to make fusion between autophagosomes and lysosomes. As shown in Figure 4, the two markers colocalized (yellow coloration) in the Rapamycin-treated cells but not in virus-infected cells. These results suggest that the virus prevents maturation of autophagosomes in the infected cells. It is also noteworthy that large lysosomal bodies became visible in the Rapamycin-treated as well as in the virus-infected cells.

Effect of modulation of autophagy on HHV-6 replication

We determined the effects of the HHV-6-induced autophagy on the viral replication. For this purpose, we treated the mock and virus-infected cells with vehicle (DMSO), Rapamycin or 3-MA. On day 10 post-infection, we examined the cultures under an inverted microscope. As shown in Figure 5, Rapamycin inhibited HHV-6-induced cell death but slightly increased size of the cells compared to the mock-infected cells. The treatment with 3-MA, on the other hand, did not prevent cell death but increased the number of large balloon-shaped cells with condensed chromatin. We collected the cells, washed them with PBS and lysed them to perform Western blots for a late viral antigen using a monoclonal antibody 2D6, which specifically binds with a late viral glycoprotein complex, gp 82/105 (41). As shown in Figure 6, the treatment with 3-MA significantly decreased expression of the viral late antigen. Rapamycin, on the other hand, increased this expression in the virus-infected cells.

The culture supernatants obtained from the Rapamycin and 3-MA-treated cells described above were clarified from cell debris by centrifugation at 1000xg. The clarified supernatants were titrated for HHV-6 genomes by real time PCR as described in the Materials & Methods' section. Interestingly, the treatments with Rapamycin decreased the numbers of viral genomes released into the culture medium, whereas 3MA increased them (Figure 7). We also examined the cells under electron microscope (Figure 8). More viral particles were seen inside Rapamycin-treated cells. However, more of them were seen being released from the 3MA-treated cells.

Taken together, these data suggest that induction of autophagy with Rapamycin reduces HHV-6 replication and/release of virions from the virus-infected HSB-2 cells. Preventing autophagy, on the other hand, increased viral replication and/or release of virions from the infected cells.

Discussion:

Autophagy is considered as a host defense to protect cells from cancer, starvation and invading pathogens. A low-grade autophagy occurs in cells as a kind of janitorial process by which old and functionally compromised organelles e.g., mitochondria, long-lived, mis-folded and abnormal aggregates of cellular proteins are degraded and recycled (1, 2). Autophagy-deficient animals are prone to higher incidence of various cancers. However, excessive autophagic response may also cause cell death. A variety of viruses including Herpesviruses are known to induce autophagic response in host cells. From the host's point of view, it may be considered as a response to protect the host by sequestering and degrading foreign. As a counter defense, viruses have evolved a variety of different strategies for evading this host response; reviewed in (42). For example, HCV induces autophagic vacuoles via unfolded protein response but prevents fusion between autophagosomes and lysosomes (formation of autolysosomes) and degradation of the autophagosomal contents. Inhibiting autophagy in the host cells prevents replication of the virus. It was also demonstrated that the virus uses autophagic membranes for translating incoming viral RNA. Thus HCV represents a typical virus that modifies host's autophagic response and exploits it for its own replication (43-45). Another virus, HSV-1, encodes a viral protein ICP34.5 with the potential to inhibit autophagy (46, 47). Interestingly, HCMV, a virus that belongs to the same Herpesvirus subfamily as the HHV-6, also is known to modulate this process. The virus does so by a mechanism, which is different from the one used by HSV-1. It activates mTOR, a potent inhibitor of autophagy (33); reviewed in (42). Thus different viruses use different mechanisms to evade autophagic response of the host. However, to date no study has been published concerning this process in HHV-6 infection.

We show here for the first time that HHV-6 induces autophagic response in human cells. The appearance of autophagic vacuoles can be seen as early as three days post-infection and they gradually enlarge as the infection progresses. However, the virus-induced autophagosomes remain smaller than those seen in the cells treated for 24 hours with Rapamycin. Our Western blot results, nevertheless, show that the

LC-3-II to LC-3-I ratio is much higher in the Rapamycin-treated, virus-infected cells compared with the Rapamycin-treated cells (Figure 2). These ratios serve as a measure of autophagy in the eukaryotic cells. These data suggest that concerning autophagy, the effect of Rapamycin and the viral infection is additive in these cells. Thus, not surprisingly, the treatment of the virus-infected cells with Rapamycin increases, and with 3MA decreases autophagic vacuoles in HHV-6-infected cells.

In order to evade host's autophagic response, one of the strategies used by viruses is to inhibit maturation of autophagosomes (8, 9); reviewed in (42). To achieve this end, viruses often inhibit fusion between autophagic vacuoles and lysosomes. A good example in this case is that of HCV (43-45). We determined whether HHV-6 was also inhibiting the fusion between autophagosomes and lysosomes. For this purpose, we stained cells intracellularly with LC-3 and LAMP-1, which stain autophagosomes and lysosomes, respectively and examined them under a confocal microscope to see if the two colors colocalize or not. In the case of the mock-infected cells that were treated for 24 hours with Rapamycin, the stains colocalized with each other creating a hybrid (yellow) color, whereas the two colors did not colocalize with each other in the virus-infected cells. These data suggest that the infection prevents fusion between autophagosomes and lysomes.

It is noteworthy that autophagy not only represents an innate defense mechanism of the host to sequester and eliminate invading pathogens, it also plays an important role in inducing a pathogen-specific adaptive immune response by promoting antigen presentation. The antigens sequestered in autophagosomes can be presented to naïve T cells via MHC class I (cross presentation) and MHC class II molecules (48); reviewed in (49). By evading autophagy, viruses can also prevent their detection by immune surveillance of the host.

Finally we determined the effects of two autophagy modulators, Rapamycin, and 3MA, on viral replication in HHV-6-infected HSB-2 cells. Rapamycin prevented cell death in the virus-infected cells. However, the infected cells became relatively bigger than the untreated cells. In the case of 3MA, more cells death was observed in the virus-infected cells compared to the untreated virus-infected cells. Western

blots on these cell lysates, developed for a late viral antigen, showed a slight increase in expression in the Rapamycin-treated cells and a slight decrease in 3MA-treated HHV-6-infected cells compared to the virus-infected cells without any treatment (Figure 6). When we examined the numbers of viral genomes present in the culture supernatants of these cells, Rapamycin-treated cells released less viral particles into media compared with the vehicle-treated cells. However, the 3MA-treated cells increased the numbers of released virions into culture medium. Taken together, these data suggest that the treatment with 3-MA increased release of virions into culture medium. This may have led to decreased expression of the late viral antigen inside these cells. The treatment with Rapamycin appears to have decreased release of virions into culture medium, despite an increased expression of the late viral antigen inside these cells. These results can be explained by assuming that autophagy is an antiviral response. Its inhibition by 3MA decreases this response and more viral replication occurs and more virions are released into the medium. On the other hand, Rapamycin augments this response, which the virus cannot counter. Therefore, Rapamycin exerts inhibitory effects on viral replication/morphogenesis and results in decreased numbers of the viral particles into culture medium. However, other explanations are also possible as the autophagy modulators used in our study also exert effects on cells that are independent from their effects on autophagy. For example, Rapamycin inhibits mTOR, which represents the most important hub in the cellular metabolism (36). Similarly, 3MA inhibits class III PI-3K, and has also been shown to exert effects on cells independently from autophagy (50, 51). Further studies are needed to know that the effects of these modulators on the viral replication are mediated entirely through their effects on autophagy. As mentioned above, viruses differ from each other in evading, modifying and exploiting host's autophagic responses; reviewed in (42). Therefore, it is not surprising that autophagy modulators affect their replication differently. For example, Rapamycin, which inhibits HHV-6 replication, has been shown to enhance HIV-1 replication (52).

In brief, we show here for the first time that HHV-6 induces an autophagic response in human cells. However, the infection also inhibits fusion between autophagosomes and lysosomes. Furthermore, we show that Rapamycin inhibits, and 3MA increases viral replication and release of virions into culture media.

Acknowledgements:

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Figure Legends

Figure 1: HHV-6 induces autophagy in HSB-2 cells. Mock and HHV-6 infected HSB-2 cells (day 6 post infection) were stained intracellularly with FITC-conjugated anti-LC-3 antibody and DAPI. The stained cells were examined under a confocal microscope. As a positive control for autophagy, HSB-2 cells were treated for 24 hours with Rapamycin (1ug/ml). Green coloured aggregates of LC-3 in the confocal images represent autophagic vacuoles.

Figure 2. HHV-6 infection induces cleavage and lipidation of LC-3. In order to detect cleavage and lipidation of LC-3, Western blots were performed on lysates from mock (M) and HHV-6 (H) infected HSB-2 cells using an anti-LC3 antibody. The subscript R indicates treatment with Rapamycin. In the Western blot panels, the upper bands indicate uncleaved and unlipidated LC3 (LC-I) and lower ones indicate cleaved and lapidated LC-3 (LC-II). R values below the Western blot lanes indicate ratios between LC-3-II and LC-3-I band intensities for each lane.

Figure 3. Effects of Rapamycin and 3-MA on HHV-6-induced autophagy. HSB-2 cells were infected or mock-infected with HHV-6 with or without the addition of Rapamycin or 3-MA. On day 8 post-infection, the cells were harvested, stained intracellularly with FITC-conjugated anti-LC-3 antibody and DAPI. The stained cells were examined for autophagic vacuoles under a confocal microscope. The cells with autophagic vacuoles were counted in one hundred cells. The Figure shows data from three independent experiments.

Figure 4. HHV-6 inhibits fusion between autophagic vacuoles and lysosomes. In order to examine fusion between autophagic vacuoles and lysosomes, the cells were stained intracellularly with FITC-conjugated anti-LC-3 and Rhodamine-conjugated anti-LAMP-1 antibodies. The stained cells were examined under a confocal microscope. The Figure shows typical images from mock-infected, HHV-6-infected cells on day 8 and day 12 as well as from Rapamycin-treated cells. Note co-localization of autophagic vacuoles and lysosomes occurs in Rapamycin-treated cells (yellow colouring), but not in HHV-6-infected cells.

Figure 5: Effects of autophagy modulators on HHV-6 infection in HSB-2. The cells were infected or mock-infected with HHV-6 and incubated in the presence of vehicle, Rapamycin or 3-MA. The cultures were examined daily and the photomicrographs were taken day 10 post-infection. A) Mock-infected cells. B) HHV-6 infected cells. C) HHV-6-infected cells with Rapamycin. D) HHV-6-infected cells with 3-MA. Note lack of any cytopathic effect (CPE) in HHV-6-infected plus Rapamycin-treated cells.

Figure 6. Effect of autophagy modulators on viral protein expression. The cells were infected with HHV-6 and treated with the reagents as described in the legend to Figure 5. On day 10, the cells were lyzed and Western blots for the expression of a late viral glycoprotein complex (gp 82/105) were performed using a specific monoclonal antibody 2D6. The letters M and V designate mock and HHV-6-infected cells, respectively. The subscripts M and R refer to treatment with 3-MA and Rapamycin, respectively. The table below the Western blots shows band intensities in pixels as well as their ratios with actin band intensities.

Figure 7. Effects of autophagy modulators on release of virions into culture media. The numbers of viral genomes in the culture supernatants (described in the legend to Figure 5) were determined by a commercial real time PCR kit. The numbers represented viral copies per ml in the supernatants.

Figure 8. Electron micrographs of the cells. The virus-infected and mock-infected cells were cultured in the presence of vehicle, Rapamycin or 3MA. On day 10, the cells were processed for electron microscopy and photomicrographs were taken. The virus-infected cells show elongated processes, vacuoles and some lipid-containing bodies. These features were either absent or minimally present in the mock-infected cells. HHV-6-infected and Rapamycin-treated cells show a lot of membrane bound vacuoles and some para-crystalline-inclusions in some cells. The virus-infected and 3MA-treated cells have less membrane-bound vacuoles and

para-crystalline inclusions. Note more virions in a multivesicular body for release in these cells. These cells also show nuclear degeneration and alterations in the nuclear membrane. Numerous nuclear membrane extrusions and membrane-bound vesicles can also be seen in the cytoplasm.

FIGURES

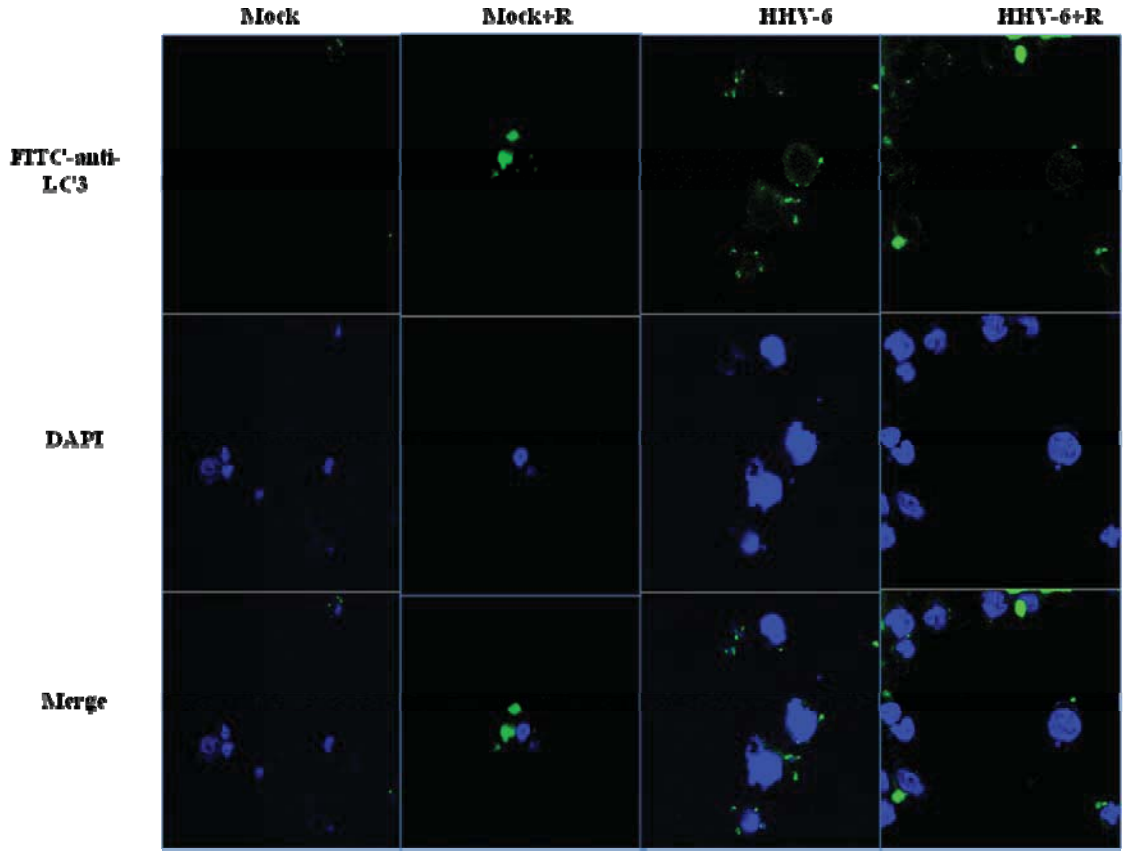
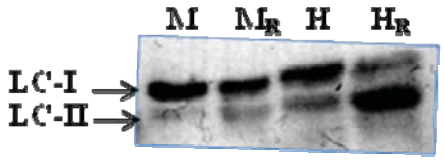


Figure 1



R	0.48	0.68	0.76	1.60

Figure 2

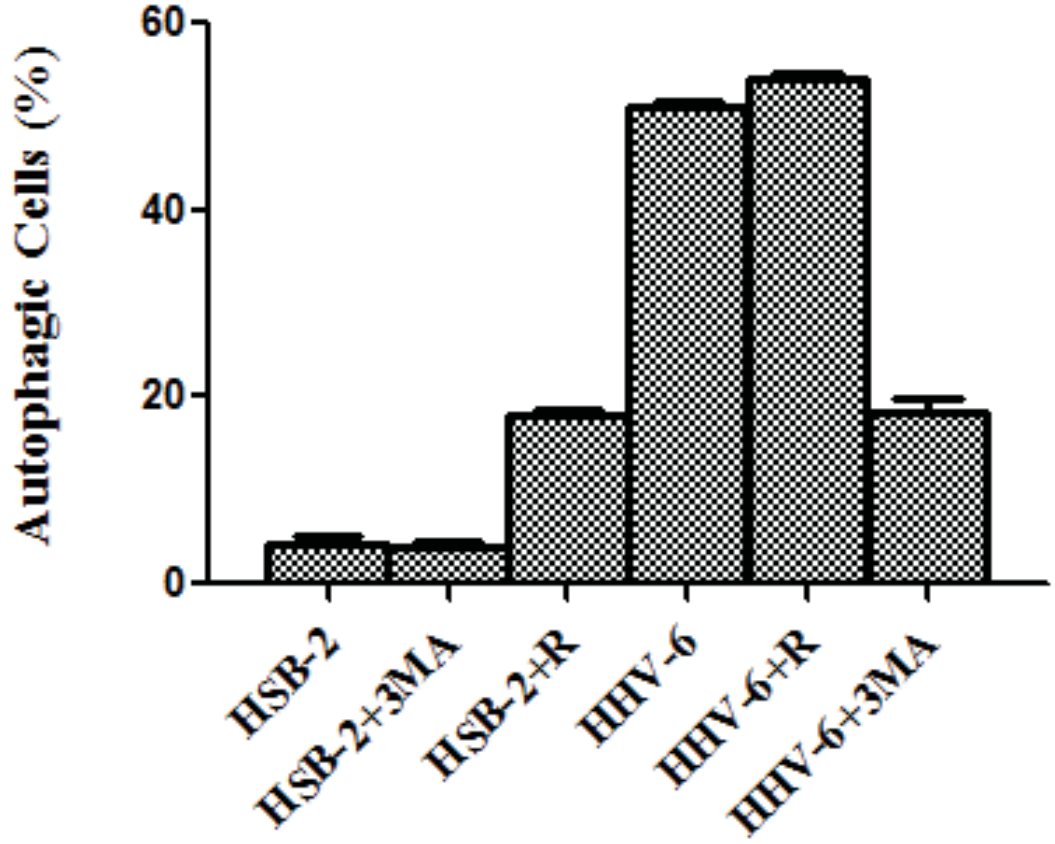

Autophagic Cells (%)
60
40
20
0
HSB-2
HSB-2+3MA
HSB-2+R
HHV-6
HHV-6+R
HHV-6+3MA

Figure 3

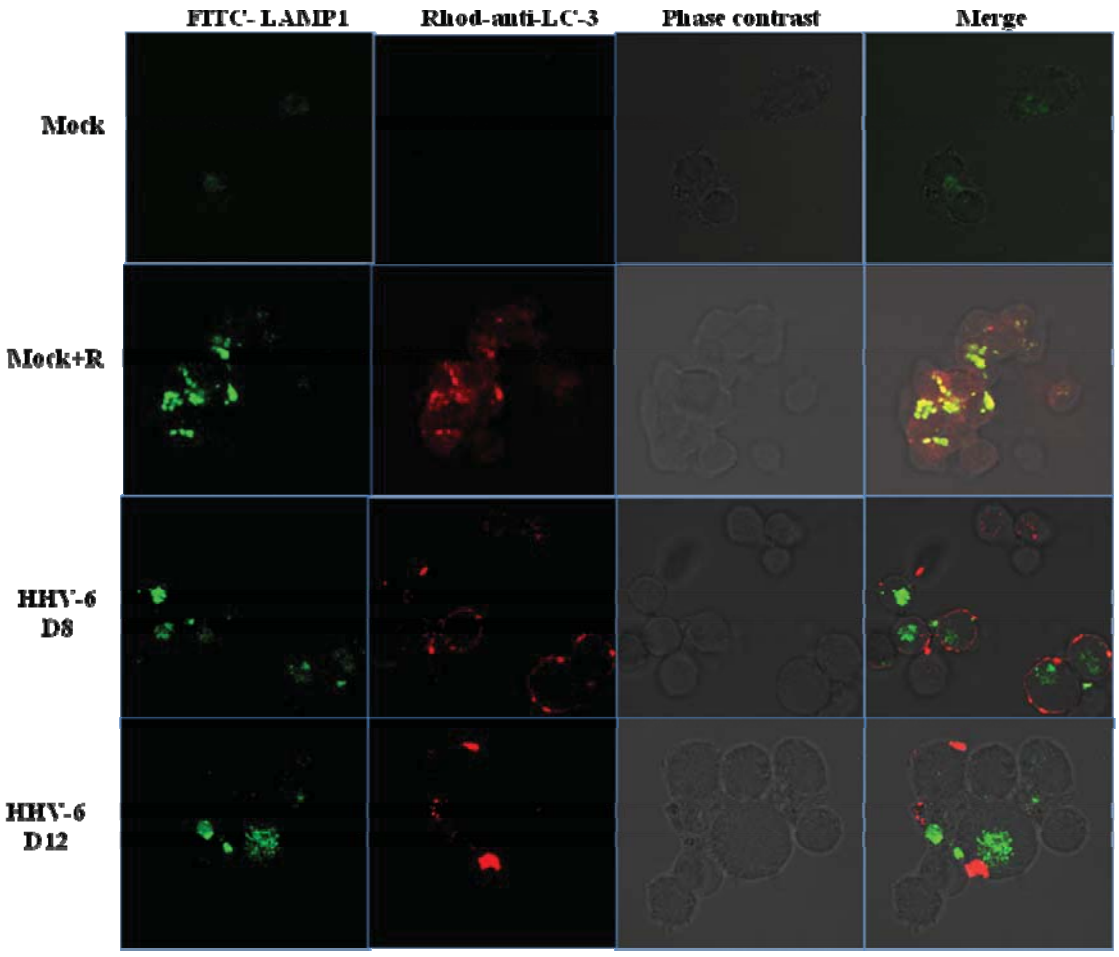

FITC- LAMP1
Rhod-anti-LC-3
Phase contrast
Merge
Mock
Mock+R
HHV-6
D8
HHV-6
D12

Figure 4

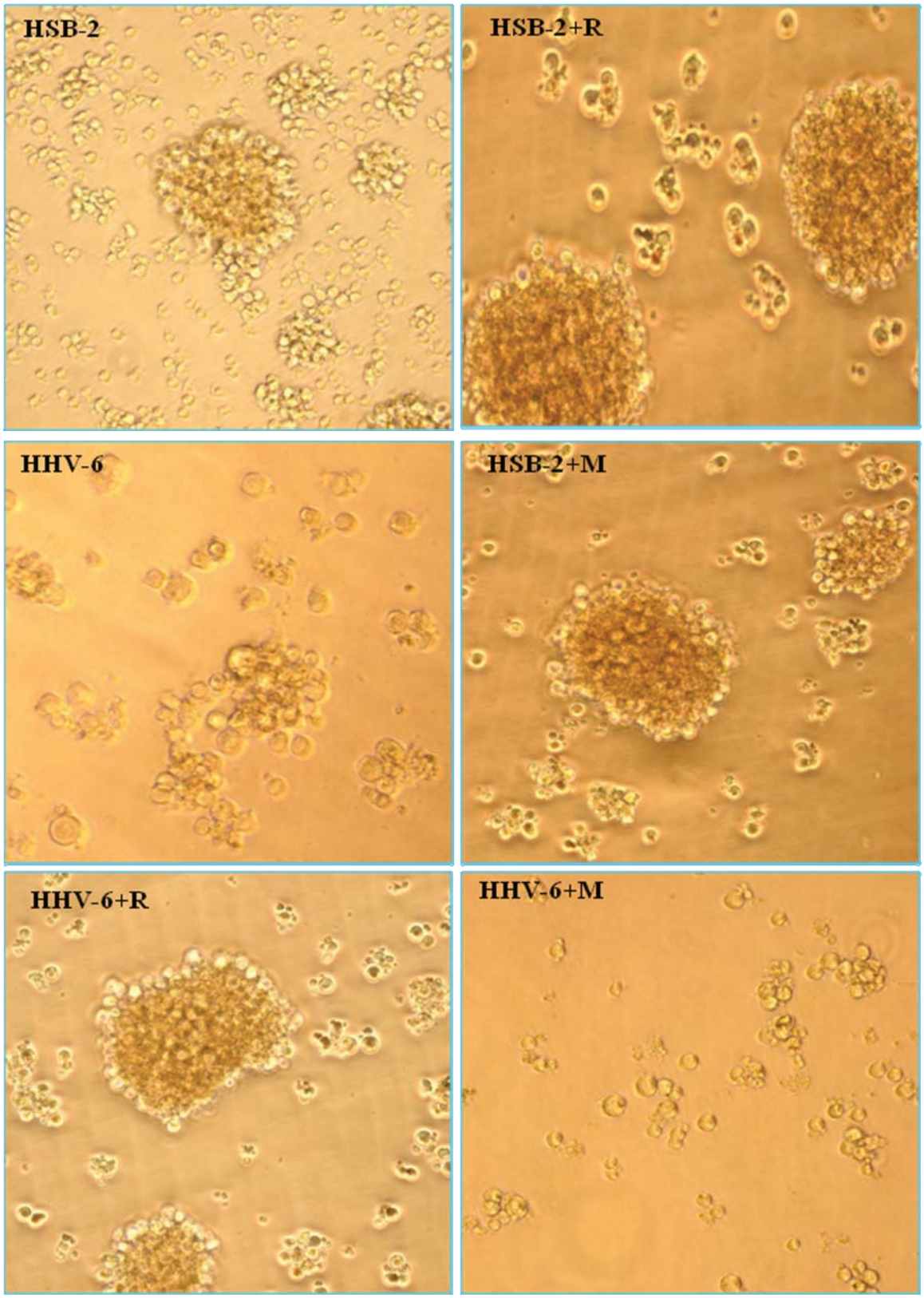
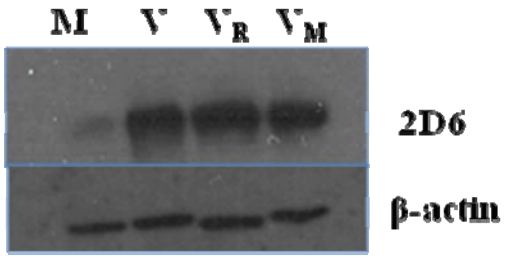


Figure 5



R 0.88 1.09 1.14 1.12

Figure 6

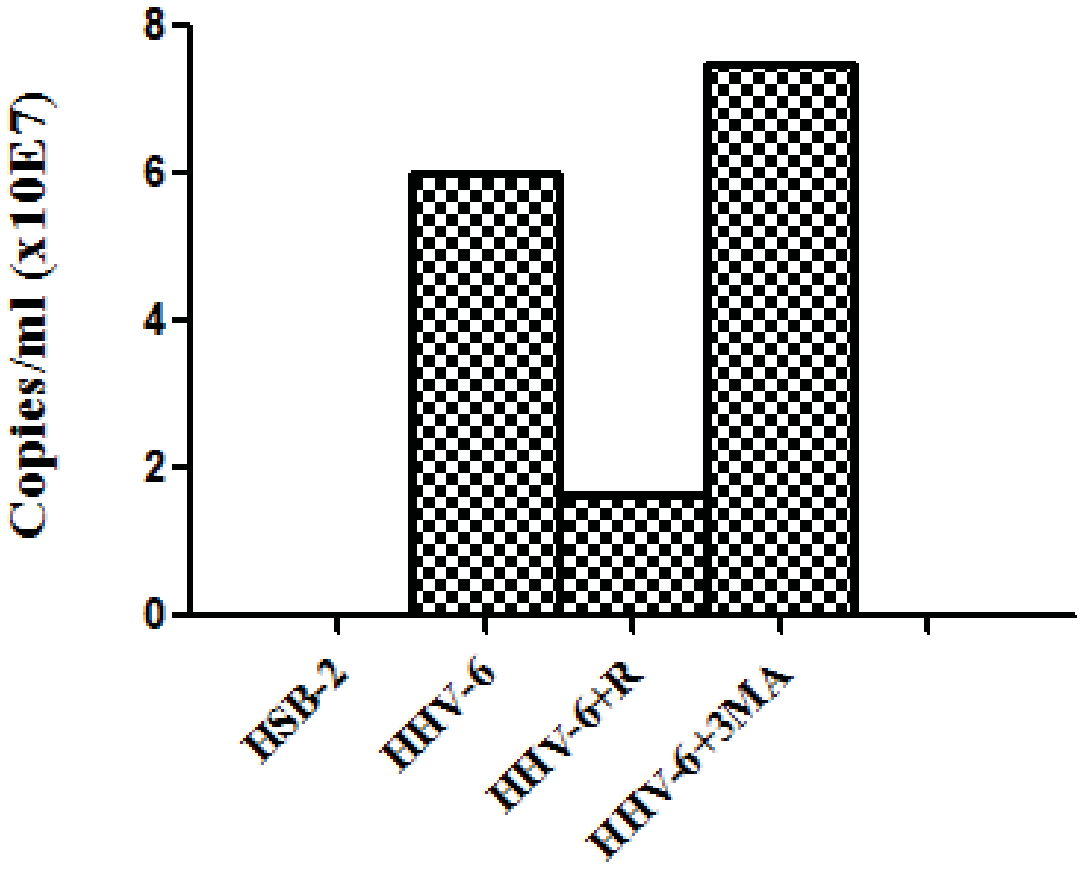


Figure 7

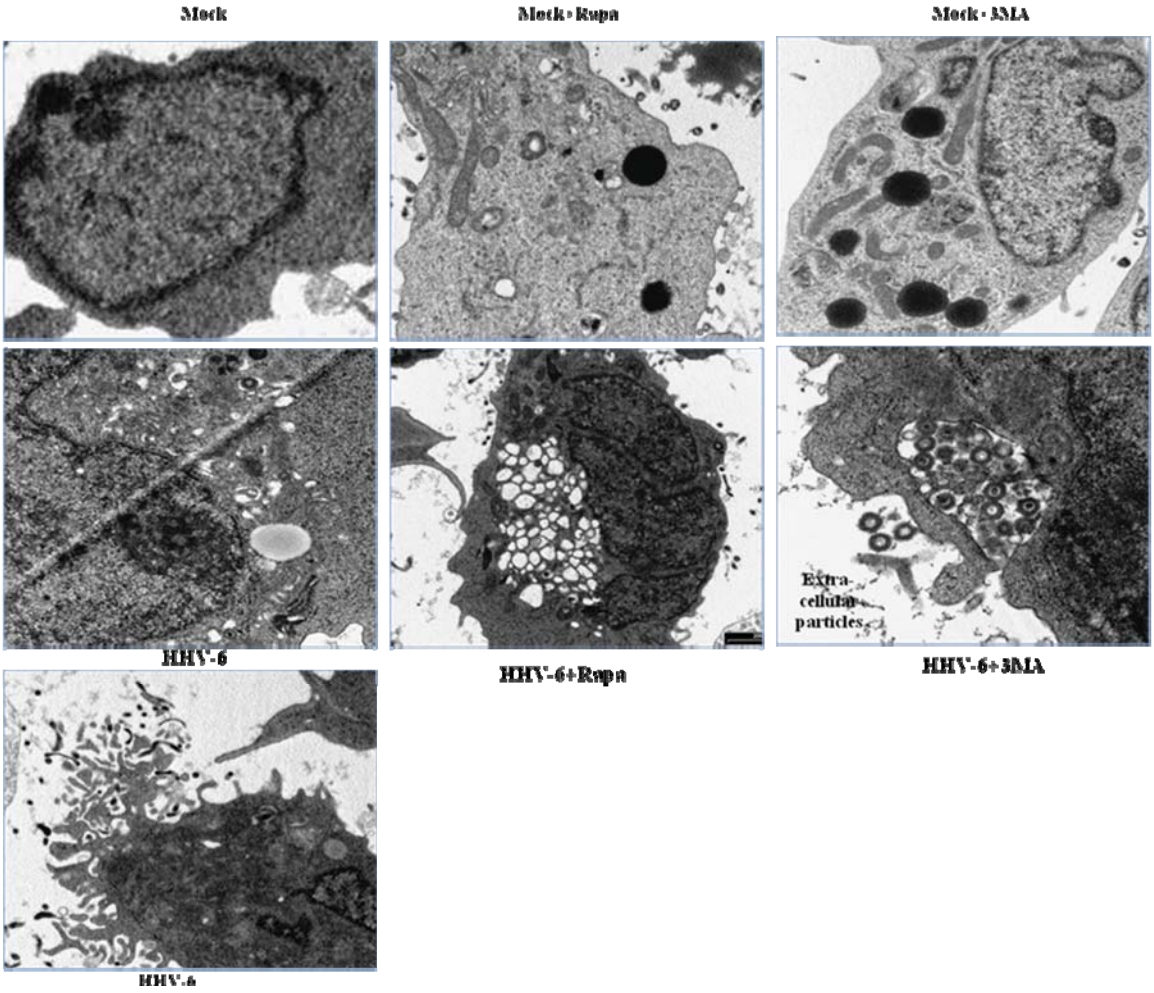

Mock
Mock+Rapa
Mock+3MA
Extra-cellular particles
HHV-6
HHV-6+Rapa
HHV-6+3MA
HHV-6

Figure 8

Résultats 3: Depletion and inhibition of p53 induces cell death in human cells that can be prevented by inhibitors of autophagy and necroptosis.

Article 4: Olfa Debbeche and Ali Ahmad

Résumé: P53 est un gène suppresseur de tumeur. Son produit, la protéine p53, est rapidement dégradée et elle est donc exprimée à des niveaux faibles dans les cellules normales. La protéine est stabilisée et activée en réponse à différents stimuli de stress. Les p53 activées peuvent provoquer l'arrêt du cycle cellulaire, la sénescence ou la mort cellulaire (apoptose) dans les cellules humaines. On sait peu concernant la fonction des niveaux basaux de cette protéine dans les cellules humaines. Nous rapportons ici que l'inhibition de l'expression de p53, par siRNA ou par un inhibiteur pharmacologique, conduit à la mort cellulaire dans les cellules humaines. Cette mort cellulaire pourrait être évitée par des inhibiteurs d'autophagie et de nécroptose et non pas par des inhibiteurs d'autophagie ou par les charognards des espèces oxygénées réactives. Nos observations rajoutent à la complexité des fonctions médiées par p53 et mettent en évidence le rôle de son niveau d'expression basale dans la survie cellulaire.

Depletion and inhibition of p53 induces cell death in human cells that can be prevented by inhibitors of autophagy and necroptosis

Olfa Debbeche and Ali Ahmad

Running Title: A prosurvival function of basal p53

Abstract

P53 is a tumor suppressor gene. Its product, p53 protein, is rapidly degraded and hence is expressed at low levels in normal cells. The protein becomes stabilized and activated in response to several stress-inducing stimuli. Activated p53 may cause cell cycle arrest, senescence or cell death (apoptosis) in human cells. Little is known concerning the function of basal levels of this protein in human cells. We report here that suppression of *p53* gene expression by siRNA as well as its pharmacological inhibition leads to death in human cells. The cell death could be prevented by inhibitors of autophagy and necroptosis, but not by a broad spectrum caspase inhibitor (also called pan caspase inhibitor) or a scavenger of reactive oxygen species. Our observations add to the complexity of the p53-mediated functions and highlight a role of its basal level expression in cell survival.

Key Words: Apoptosis, Autophagy, Necroptosis, Nutlin-3, p53, Pifithrin

Introduction

P53 is a tumor suppressor gene that has been dubbed as "the guardian of the genome" as well as "cellular gatekeeper" ((1); reviewed in (2)). The gene product, p53, is stabilized and activated in response to several stress-inducing stimuli, e.g., DNA damage, hypoxia, oncogenic stress, starvation, stalled DNA replication, viral infections, etc. Activated p53 regulates transcription of a large number of cellular genes via its transcription-activation domain and also exerts some effects independent of its effects on gene transcription (3). Consequently, p53 activation may lead to cell cycle arrest, senescence or apoptosis. These outcomes of the p53 activation are context-dependent and may differ with the cell type and cellular microenvironment. Notably, p53 is the most mutated protein in human cancers. About one half of the human cancers lack functional p53 gene, and others are often defective in p53-induced signaling pathways (4-6).

Viral infections, in general, induce activation of p53, which may stop cells in G1 phase of the cell cycle, cause cell death or senescence. These results may not be very conducive to viral replication. Therefore, viruses usually encode one or more proteins that inactivate p53 response directly or indirectly (reviewed in (7, 8)). For example, the Adenovirus type 5 encodes a protein E1B (55 kD), which inhibits transcriptional activity of p53. The viral protein, in association with another viral protein E4-Orf-6, also causes enhanced proteasome-mediated degradation of this tumor suppressor protein (reviewed in (9)). It was hypothesized that E1B-deleted adenoviruses would not be able to inactivate p53, and hence would replicate only in p53-mutant cancer cells and kill them preferentially. This led to the development of oncolytic adenoviruses (e.g., Onyx-015). Interestingly, the viruses have shown efficacy clinically and have been approved for treating p53-mutant human cancers. Ironically, the anti-tumor response of the virus was found to depend on a stress response induced by the late viral mRNA rather than p53 status of the killed tumor cells ((10, 11); reviewed in (12)). Building upon this principle, a variety of different oncolytic tumor viruses has been developed that preferentially target, and proliferate better, in p53-mutant cells (13).

Our laboratory is interested in understanding the pathogenesis of HHV-6. The virus is known to infect and replicate best in HSB-2 cells, a T lymphoblastic leukemic cell line (14, 15). The cell line was derived from the peripheral blood of an 11 year old male Caucasian suffering from acute lymphoblastic leukemia. The cell line was developed after their eight passages in new-born Syrian hamsters ((ATCC CCL 120.1; (16)). The cells are diploid (2n), harbour wild type *p53* but mutant *pRb* (17).

The HSB2 cells respond to DNA damage caused by gamma irradiation and DNA damaging drugs (cisplatin, etoposide, etc) by activating p53, inducing expression of p21 and undergoing G1 arrest and apoptosis. The cells grow in suspension (doubling time 36.2 hours), and are very susceptible to changes in pH and growth factors. Extra care is needed to maintain the cells in culture. The cell line is highly radiosensitive and undergoes apoptosis very rapidly (15). Despite being the most permissive cell line for HHV-6 replication, the virus takes a relatively long time (10-12 days) to complete its replication and induce cytopathic effects in these cells. This makes many experiments difficult to perform. For example, cell cycle synchronisation becomes practically useless when studying effects of the virus on cell cycle in this cell line. Furthermore, one has to follow the infection for 12 days to study kinetics of the viral replication or of any given cell function and parameter.

As mentioned above, HSB-2 cells express wild type p53, are highly radiosensitive and undergo apoptosis rapidly. It has also been demonstrated that HHV-6 infection induces p53 stabilization and activation in the virus-infected cells (18-21). The virus is known to encode two viral proteins, DR-7 (Orf-1) and an Orf14-encoded protein. The proteins bind and inactivate p53 (22, 23). Despite this, p53 has been shown to be activated (phosphorylated at Ser 15, Ser 20, and Ser 392) and capable of binding DNA in the virus-infected cells (19, 20).). Furthermore, the virus-induced p53 activation has been shown to suppress HHV-6 replication in these cells (21).

Under these circumstances, we hypothesized that inhibition of p53 expression or its inactivation by pharmacological inhibitors may promote viral replication and

shorten the viral life cycle. Quite unexpectedly, we found that the cells, in which p53 expression was inhibited by specific siRNA or was inactivated by a pharmacological inhibitor, underwent enhanced cell death, which could be inhibited by an inhibitor of autophagy or of necroptosis , and not by a scavenger of reactive oxygen species (ROS) or by a pan-caspase inhibitor. The results from this study show that basal constitutive levels of p53 exert pro-survival effects in human cells.

Materials and Methods

Cell culture and reagents

The HSB-2 and CEM cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2.5 mM L-glutamine, and antibiotics (100 units per ml of penicillin from Novapharm, Toronto, Canada; 2 μg per ml of gentamycin from Schering, Pointe Claire, Canada and 2.5 μg per ml of fungizone from Squibb, Montreal, Canada) as described earlier (24). Both these cell lines are of T cell origin and have derived from leukemic patients. HSB-2 harbors wild type *p53* gene, whereas the gene in CEM is mutant and transcriptionally inactive (15, 17, 25). Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors, activated in vitro with Phytohaemagglutinin (PHA; 10 μg per ml; Sigma-Aldrich) and 10 units per ml of IL-2 (Roche) in the culture medium. After 72 hours, PHA blasts were used in the experiments following published procedure (26).

The reagents used in the study were: N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK; a cell permeable pan-caspase inhibitor; Calbiochem; Catalog #627610), 3-Methyladenine (3MA; an inhibitor of autophagy; Sigma-Aldrich; #M9281), Rapamycin (an inhibitor of mTOR and inducer of autophagy; Cell Signalling Technology; Catalog #9904), Pifithrin (PFT; a p53 inactivating agent; Sigma-Aldrich; Catalog #P4359), Nutlin-3a (a p53-stabilizing agent; Sigma-Aldrich; Catalog #N6287), Bafilomycin A1 (an inhibitor of vacuolar proton pump; Sigma-Aldrich; Catalog #B1793), 4, 6-diamidino-2-

phenylindole (DAPI; a DNA-staining agent; Sigma-Aldrich), Necrostatin-1 (a specific allosteric inhibitor of RIP-1 and necroptosis; Tocris Bioscience; Catalog #2324) and N-acetyl-L-cysteine (NAC; a precursor of glutathione and a powerful anti-oxidant; Sigma-Aldrich; Catalog #9165) . Antibodies used included mouse anti-human β-actin (Sigma; Catalog # A5441), Rabbit anti-LC-3 antibodies (MBL; catalog #PM036 and catalog #M152), and Mouse anti- humanLC-3 antibody (MBL); FITC-conjugated goat anti-mouse (Biolegend; Catalog #405305).

Inhibition of p53 expression with siRNA

To inhibit p53 expression, we used the Gene Suppressor System (Imgenex Corporation, San Diego, CA; Catalog # IMG-701; Ref (27). It is a plasmid-based system that generates siRNA under the U6 promoter to knock down p53 expression upon transfection in mammalian cells. The siRNAs are expressed as 50 nucleotide fold-back stem-loop structures, which are processed to generate double stranded siRNA of less than 21 nucleotides. The plasmid-based siRNA expression leads to long-term suppression of the specific gene. The kit also contained IMG-700-06 as the negative control plasmid. It expresses the dsRNA with scrambled sequences.

DNA Transfection

As per instructions of the manufacturer of the siRNA plasmid kit, 2.5 ug of the plasmid DNA (siRNA or control vector) was transfected into one million cells using the DMRIE-C Reagent (Invitrogen Catalog #10459-014).

Western blots

The expression of p53 in the vector-transfected cells was examined using Western blots using p53-specific mouse anti-human p53 (Santa Cruz; catalog #Sc-126). The Western blots were performed as described previously (24, 28). Briefly, 1-2 million cells were washed with PBS and lysed in a lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 2 mM DTT and boiled for 15 seconds. The particulate matter was removed from the lysates by centrifugation at 14000g for 30 minutes at room temperature and their protein concentrations were determined with a commercial kit (CBA kit from Pierce, catalog #23225). Thirty to fifty ug of

the cell lysates were loaded on 10% SDS-PAGE. The resolved proteins from the gel were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore Corp., Bedford, MA) using a semi-dry transfer system (Bio-Rad). The p53 bands on the membranes were revealed by using a commercial Western blot kit (Vectastain ABC-AMP; Vector Laboratories, AK-6601). The molecular weight markers (Rainbow; Amersham, Bioscience; catalog #RPN800) were run in one lane on each gel to locate positions of the proteins with known molecular weights.

Virus stock:

In some experiments, we infected cells with HHV-6. For this purpose, we used GS strain of the virus (A variant). The virus was grown in HSB-2 cells and was prepared as described earlier (29). The supernatants collected from uninfected HSB-2 cells were used for mock infections. The viral stocks used had a titre of 5-9x10^5 $TCID_{50}$ per ml. In order to determine whether cells have been infected with the virus, they were stained intracellularly with a virus-specific monoclonal antibody 2D6, which specifically binds to a late viral antigen, the gp85/102 complex (30). The cells were washed, incubated with FITC-conjugated goat anti-mouse IgG for 30 minutes, washed again with PBS containing 0.5 % bovine serum albumin and examined by flow cytometry.

Detecting Autophagic cells

Two methods were used to detect autophagic cells: 1) the cells were fixed and permeabilized using a kit (Cytofix; BD Pharmingen), and stained with an FITC-conjugated anti-LC-3 antibody (MBL, catalog #PM036) and DAPI. The stained cells were examined under a confocal microscope. The autophagic cells showed small LC-3-positive vesicles (puncta) in their cytoplasm. Normal cells, on the contrary, showed faint and diffuse staining for LC-3. No vesicles were seen in these cells. The % of autophagic cells with LC-3 puncta in a given sample was determined by observing 100-120 cells. 2) The extent of autophagy in the cells was determined from ratios of LC-3II to LC-3I bands on Western blots.

Detection of apoptosis

A commercial kit (BD Pharmingen) was used to detect the % age of apoptotic cells. The kit uses staining of the cells with FITC-conjugated annexin V and

propidium iodide (PI). The stained cells were washed with PBS, resuspended in FACS buffer and examined by flow cytometry (FACSCalibur; B&D).

Statistical analysis

The results were compared between different treatment groups using Student's T test. The differences at $p \leq 0.05$ were deemed significant. *, **, and ***, statistically significant differences between indicated means (P0.05, P0.01, and P0.001, respectively).

Results

Suppression of p53 expression causes cell death in human cell lines

As described in the section on Materials & Methods, we transfected the human T cell line HSB-2 with an expression plasmid encoding p53-specific siRNA or with a control plasmid encoding siRNA with specificity for no known human gene. After 24 hours of the transfection, we verified the expression of p53 in the transfected cells by Western blots. As shown in Figure 1, the transfection of the cells with p53-siRNA vector, but not with the control vector, caused a marked decrease in the expression of p53. Almost similar results were obtained when the known to have a transcriptionally mutant p53 gene ADDIN EN.CITE <EndNote><Cite><Author>Massumi</Author><Year>2006</Year><RecNum>516</RecNum><DisplayText>(25)</DisplayText><record><rec-d for cell death 24 hours and 48 hours later. As shown in Figure 2, the p53-specific siRNA plasmid transfected cells underwent significantly more cell death at both time points as compared with the control vector-transfected cells. These data suggest that a decreased expression of p53 caused cell death in HSB-2 cells.

The inhibitor of autophagy, 3MA, reduces cell death in p53-suppressed human cells

In order to determine whether the cell death caused by the suppression of p53 gene expression by p53-specific siRNA could be modulated by 3-MA (an inhibitor of autophagy), Rapamycin (an inducer of autophagy), or a pan-caspase inhibitor (Z-

VAD-FMK), we incubated the siRNA and control vector transfected cells with these regents and determined cell death 24 and 48 hours later. The results from three independent experiments are shown in Figure 3. The siRNA-induced cell death was significantly reduced but not completely inhibited by 3-MA ($p<0.05$). Another inhibitor of autophagy Bafilomycin A also had similar effects (data not shown). These data suggest that basal constitutive level expression of p53 exerts pro-survival effects, which are mediated at least in part by inhibition of autophagy. In contrast to 3MA, the caspase inhibitor and Rapamycin augmented cell death in both siRNA and control vector-transfected cells (Figure 3).

Inhibition of p53 expression induces autophagic response in HSB-2 cells

In separate experiments, we determined whether the siRNA-mediated inhibition of p53 in HSB-2 cells induces autophagy. For this purpose, we stained the siRNA transfected cells intracellularly with FITC-conjugated anti-LC-3 antibodies and DAPI, and examined them under a confocal microscope. As shown in Figure 4A, the transfection of p53-specific siRNA vector induced LC-3 aggregates (a marker of autophagy). These aggregates were not induced in the control vector transfected cells. Furthermore, the treatment of the siRNA-transfected cells with Rapamycin increased and with 3-MA decreased these aggregates. As expected, Rapamycin also induced LC-3 aggregates in control vector transfected cells. The treatment of the siRNA vector transfected cells with 3MA inhibited the autophagic response. Of note, Rapamycin induced bigger autophagic vacuoles in the siRNA transfected cells. Furthermore, 3-MA was not able to completely inhibit autophagy in these cells. The %ages of the cells undergoing autophagy in the siRNA or control vector transfected cells are shown in Figure 4B. These data suggest that a decrease in the expression of basal level p53 induces autophagy in HSB-2 cells.

Inactivation of p53 with a pharmacological inhibitor also induces cell death in HSB-2 cells

After seeing increased cell death upon siRNA-mediated inhibition of p53 expression, we sought to determine the effects of a chemical antagonist of p53, the

cyclic analog of Pifithrine (PFT). The agent is known to inhibit transcriptional activity of p53; although the exact mechanism of its action remains unknown (31). For this purpose, we incubated HSB-2 with PFT for 24 hours and determined cell death with Trypan blue exclusion. As shown in Figure 5A, the p53 inhibitor caused cell death in these cells. We also determined the effects of a pan-caspase inhibitor (Z-VAD-FMK) and an autophagy inhibitor (3-MA) on the PFT-induced cells death. Interestingly, the PFT-induced cell death was inhibited by 3-MA but not by the caspase inhibitor. Interestingly, 3MA was not able to reduce cell death in the presence of the caspase inhibitor. We also sought to determine the effects of Nutlin-3a, on HSB-2. Nutlin-3a binds HDM-2 in its p53 binding pocket and prevents its binding with, and degradation of, p53 (32). Consequently, it stabilizes and activates p53. It has been shown to induce reversible cell cycle arrest in normal healthy cells, and apoptosis/senescence in adult T cell leukemic cells carrying wild type p53 (33, 34). Interestingly, activated p53 (which would have occurred in conditions of stress) also induced death, which was enhanced by 3-MA as well as by the caspase inhibitor (Figure 5B). These data suggest that p53 stabilization and activation as well as its depletion via siRNA or antagonism with a chemical inhibitor results in cell death, probably via different mechanisms.

Inhibition of p53 expression induces death in a cell line with mutant p53

It is noteworthy that HSB-2 has wild type functional p53 (17). We tested the effects of p53-specific and control siRNA on another human T cell line CEM, which is known to have a transcriptionally mutant *p53* gene (25). The results obtained with this cell line were essentially similar to those obtained with HSB-2 (Figure 6). These data suggest that even transcriptionally inactive mutant *p53* may exert its pro-survival effects.

Pifithrin and Nutlin-3 mediate cell death that lacks apoptotic features

Although the cell death mediated by PFT or Nutlin-3a was not inhibited by a pan caspase inhibitor, we sought to verify whether the dead cells showed a key apoptotic feature: expression of phosphatidylserine in the external lamella of their

cytoplasmic membranes. For this purpose, we treated HSB-2 cells with vehicle (DMSO), PFT or Nutlin-3 and determined their staining with FITC-Annexin V and PI. The stained cells were examined by flow cytometry. The results from a typical experiment are shown in Figure 7. It is quite evident from this Figure that both PFT and Nutlin-3 cause cell death, which lacks staining with FITC-Annexin V. However, in both cases, the cells become permeable to PI; a sign of compromised integrity of the plasma membrane.

N-Acetylcysteine (NAC) does not inhibit Pifithrin-induced cell death in HSB-2 cells

P53 has been shown to exert anti-oxidant properties (2, 35). Therefore, we sought to determine whether PFT-induced cell death could be inhibited by an exogenous anti-oxidant. Towards this end, we cultured HSB-2 cells in the presence of vehicle (DMSO) or PFT in the presence or absence of NAC; a powerful anti-oxidant (36, 37). After 24 and 48 hours, we counted the number of dead cells in the microcultures by Trypan blue exclusion. As shown in Figure 8, NAC had no significant impact on PFT-mediated cell death 24 hours (A) and 48 hours post-treatment (B). Furthermore, NAC also had no effect on the Nutin-3-mediated cell death in these cells after 24 (Figure 9A) and 48 hours post-treatment (Figure 9B). These data suggest that the cell death induced by a reduced expression of p53 or by Nutlin-3a-mediated stabilization and activation is not mediated by its imbalance in redox state of the cells.

P53 antagonism induces cell death in primary human T cells

So far, we had performed experiments using continuously growing T cell lines. It was important to investigate whether antagonism of p53 via PFT also induced cell death in normal human T cells. Therefore, we isolated primary human T cells from the peripheral blood of normal healthy donors, and cultured them in the culture medium containing 10 U per ml of recombinant human IL-2. We added DMSO (vehicle) or PFT and determined cell death by the Trypan blue exclusion assay. The treatment of primary human T cells with PFT resulted in their death (Figure 10). However, the extent of the death was lower than that observed in the cell lines described above. These data suggest that primary human T cells are less susceptible to death induced by p53 antagonism.

Effect of Necrostatin-1 on PFT- α and Nutlin-3-mediated cell death

It is noteworthy that other than necrosis and apoptosis, cells can also die via another death process called programmed necrosis or necroptosis. Recent studies have shown that a pharmacological inhibitor of the Receptor Interacting Kinase (RIP)-1 can specifically inhibit necroptosis in human cells (38, 39). In order to determine whether the PFT-α- and Nutin-3-mediated cell death in HSB-2 cells involved necroptosis, we incubated the cells with PFT- α or Nutlin-3 in the presence or absence of Necrostatin-1, and counted dead cells by Trypan blue exclusion. The results are shown in Figure 11. Note a significant decrease ($p>0.05$) in cell death in the presence of the RIP-1 inhibitor.

P53 transcriptional activity is required for HHV-6 replication.

Finally, we were interested in knowing the effect of p53 antagonism on HHV-6 replication in human cells. For this purpose, we infected HSB-2 cells *in vitro* with HHV-6 (GS strain) and cultured them in the presence of vehicle (DMSO), Bafilomycin-A or PFT. In these experiments, we used lower concentrations of the drug (20 nM) that did not result in significant cell death. To monitor HHV-6 replication, we determined the expression of an early viral antigen, U27-encoded p41, using a specific monoclonal antibody (9A5D12; (40)). The cells were

incubated with the antibody after permeabilization, followed by washing, staining with FITC-conjugated goat-anti-mouse IgG and flow cytometry. The results (Figure 12) showed that p53 antagonism with PFT markedly inhibited expression of the early viral antigen in the virus-infected cells as compared with the vehicle-treated cells. These data suggest that p53 may be needed for HHV-6 replication. Furthermore, the treatment of the virus-infected cells with Bafilomycin A resulted in increased expression of the viral antigen in HHV-6 infected cells.

Discussion

Here we have shown that in two continuously growing human cell lines, whether they carry wild type or transcriptionally inactive (mutant) p53 gene, siRNA-mediated suppression of p53 gene expression or pharmacologically-induced inactivation of p53 lead to enhanced cell death. The death lacked apoptotic features (i.e., binding with annexin V), and could be inhibited by an autophagy inhibitor, 3-methyladenine, as well as by an inhibitor of necroptosis, Necrostatin-1. Necroptosis has been defined as a controlled process of cell death occurring with necrotic features (39). To the best of our knowledge, this is the first demonstration of anti-necroptotic effects of p53 expressed constitutively in human cells.

p53 is well known to play a role in regulating cell cycle, apoptosis, senescence and differentiation. The protein also affects cellular metabolism and redox state of the cells (reviewed in (2, 41). Many of its biological effects derive from its role as a sequence-specific transcriptional factor. Under physiological conditions, p53 is rapidly degraded by HDM-2, an E3 ubiquitine ligase. However, in response to genotoxic, hypoxic, metabolic and oncogenic stresses, p53 accumulates, becomes activated by a variety of post-translational modifications including phosphorylation, acetylation, sumoylation, ubiquitination, etc., translocates to the nucleus and modulates the expression of a large number of target genes. Consequently, depending upon the strength and duration of the stress and resulting DNA damage, the cells may undergo transient cell cycle arrest and repair the damage, become senescent or undergo apoptosis (5). It is increasingly being

realized that a fraction of the induced p53 localizes to cytoplasm and more importantly to mitochondria and plays an important role in cell survival (reviewed in (42). The cytoplasmic p53 has been shown to inhibit autophagy and induce apoptosis. P53 acts as a pro-apoptotic "BH-3 only" protein, and interacts with Bcl-2 and BclXL (43); the anti-apoptotic members of the Bcl-2 family. This interaction inhibits anti-apoptotic functions of Bcl-2 and BclXL. P53 can also activate the pro-apoptotic protein Bax, although the exact mechanism remains unknown. The interaction of p53 with Bcl-2 family proteins occurs via its DNA-binding domain (DBD). The domain is the hotspot for mutations. Thus the p53 with mutant DBD is unlikely to mediate this function. Furthermore, the expression of PUMA (P53-upregulated mediator of apoptosis), whose gene is induced by activated nuclear p53, is essential for induction of apoptosis by the cytoplasmic p53. PUMA releases p53 from its sequestration with BclXL in the cytoplasm (44). Since PFT-α inhibits p53-induced gene transcription, PUMA is not expressed and no apoptosis occurs.

The second function mediated by the cytoplasmic p53 is inhibition of autophagy (42). The exact mechanism of this inhibition remains unknown. The depletion of p53 via siRNA or its inhibition via PFT, therefore, prevents this autophagy inhibitory function of cytoplasmic p53, resulting in the autophagy.

Our results suggest that inhibiting p53 gene expression by siRNA or inactivating its activity with PFT inhibits the prosurvival functions of the cytoplasmic p53. Our results showed that the resultant cell death was not due to enhanced apoptosis. To the contrary, the addition of a pan-caspase inhibitor, which should have prevented cell death, had it been due to apoptosis. In fact the caspase inhibitor augmented this death. Furthermore the cells showed signs of autophagy and the cell death was inhibited by 3MA, which inhibits activation of a class III PI-3K, Vps-34; a step necessary for autophagy process (45). The death was also not inhibited by NAC, a well known ROS scavenger (36). However, the death could also be inhibited by Necrostatin-1, which induces an allosteric change in the kinase domain of the Receptor-Interacting Protein Kinase (RIP)-1. Consequently, RIP-1 cannot interact with RIP-3 and induce necroptosis. Taken together, our results suggest that basal

levels of the cytoplasmic p53 promote cell survival by preventing autophagy and necroptosis.

We could find only one report in literature in which basal levels of p53 were demonstrated to be essential for cell survival (46). The workers showed that depletion of p53 by PFT-α, deletion of the p53 gene as well as suppression of the gene expression by specific siRNA led to autophagy in a variety of human, mouse and nematode cells. They also demonstrated anti-autophagic role of p53 in eunucleated cells. The authors showed that this autophagic response promoted cell survival under conditions of hypoxia and nutrient starvation. However, they did not look for cell death in the p53-depleted cells. We show here for the first time that the depleted p53-mediated autophagic response, results in necroptosis of the cells, as it can be inhibited by autophagy inhibitor 3MA and Necrostatin-1. It is noteworthy that 3-MA is well-documented inhibitor of autophagy; it inhibits a class a III PI-3K, Vps34, which is essential for the induction of autophagosome (45). Necrostatin-1 has been shown to inhibit allosteric conformation of RIP-1, which is required for its interaction with RIP-3 and induction of necroptosis ((47); reviewed in (38)). It is noteworthy that this mode of cell death lacks exposure of phosphatidylserine on the external lamellae of the plasma membrane. Consequently the cells undergoing necroptotic cell death do not stain with FITC-Annexin V. The cell death also cannot be prevented by caspase inhibitors (38). Taken together, our results suggest that physiological levels of p53 gene expression are required for cell survival. Since, these anti-autophagic effects of p53 do not depend upon its transcriptional activity, it is not surprising that we observed them even in CCRF-CEM cells, which carry a transcriptionally inactive (mutant) p53 (25). These results are in agreement with previous ones which observed anti-autophagic effects of mutant p53 in cancer cells and correlated them with cytoplasmic localization of the protein (48). It is noteworthy that in contrast to cytoplasmic p53, nuclear p53, which becomes important under conditions of stress, induces expression of genes e.g., Sestrins, DRAM (Damage-regulated autophagy modulator) that promote autophagy. The dual role of this tumor suppressor in autophagy has been recently reviewed (2, 49).

PFT was discovered as a small-molecule inhibitor of p53 in 1999 (31). It inhibited p53-mediated transcription of target genes and prevented apoptosis. The molecule showed efficacy in reducing radiation and chemotherapy-induced sickness. The exact mechanism of its action remains unknown and likely involves inhibition of p53 translocation to the nucleus. Since its discovery PFT has been shown to protect cells from apoptosis from a variety of stress-inducing stimuli ((50, 51); reviewed in (52)). As mentioned above, we have shown here that PFT-mediated depletion of basal levels of p53 in two cancer cell lines results in their death. These results are supported by an earlier publication (46). Interestingly, the p53 inhibitor also has some biological effects, which are independent of its effects on p53 (53, 54). Furthermore, the molecule is toxic at higher (> 30 μM) concentrations, and has been shown to kill certain tumor cells without affecting p53 expression or of any of its target genes (55). Although PFT may be useful in reducing toxic effects of p53 activation in chemotherapy and radiotherapy, loss of p53 function may predispose individuals to enhanced tumorigenesis. In fact it has been shown to interfere with DNA repair and induce chromosomal instability in vitro in human cells (51).

Nutlin-3a is another small molecule that binds HDM-2 and prevents its interaction with the transcription activation domain I of p53. Consequently, p53 is not degraded and accumulates in the cells (32). This accumulation is accompanied by p53 activation and cell death. The molecule has been shown to cause death and/or senescence in a variety of cancer cells with wild type p53, and acts in synergism with chemotherapy ((33, 56, 57); reviewed in (58)). In some virus-induced cancers where p53 pathway remains intact, activation of p53 by targeting its inhibitor MDM2 leads to cell death. For example, the Peripheral Effusion Lymphoma (PEL), which is caused by the KSHV and in which p53 is largely inactivated by the viral oncoprotein, Latency-associated Nuclear Antigen (LANA), activation of p53 leads to cell death (59). The cancer cells with mutant p53 gene, however, are resistant to the effects of this molecule. In line with the previous results we observed enhanced death of HSB-2 (with wild type p53; (17)) cells but not of

CEM (with mutant p53; (25)). Interestingly, the death could be inhibited with 3-MA and Necrostatin-1, and was not due to apoptosis.

One of many strategies to control cancer is re-instating p53 functions. In this regard, MDM-2 (or HDM-2 in humans) inhibitors (like Nutilin-3a) could be useful in restoring this function in cancers with wild type p53. However a majority of the cancers carry a mutant p53 and/or are defective in important p53-mediated signalling pathways (4-6, 60). So this strategy cannot be used in such cancers. The introduction of wild type p53 gene in these cancer cells is an alternate strategy. However, it is technically challenging. The use of p53 inhibitors like PFT may open up new possibilities of cancer therapy. Interestingly, the combined use of Nutilin-3a and PFT was reported to cause enhanced cell death in chronic lymphocytic leukemic cells (33).

We also show here that addition of PFT to the cultures of HHV-6-infected cells inhibits replication of the virus, when judged by the expression of an early viral antigen. Taken at face value, the results suggest p53 may be needed for replication of the virus. Interestingly, a previous study reported increased viral replication when the virus-infected cells were treated with PFT (21). However, the workers did not perform experiments in HSB-2 cells. They also used a non-cyclic form of PFT. This non-cyclic form is rapidly converted into the cyclic form used in our study. As mentioned above, PFT has also been shown to exert biological effects independent of the p53 in human cells. Further studies are needed to determine whether the differences in results in our and the previous study are due to the use of different types of cells and/or due to effects of PFT beyond p53.

In short our results provide novel insight as to the mechanism of cell death in p53-depleted/mutant cancer and normal human cells. We show for the first time the involvement of necroptosis in PFT-mediated cell death.

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Figure Legends

Figure 1. Expression of P53 in the siRNA and control vector-transfected cells. The expression of P53 in the siRNA and control vector transfected cells was determined 24 hours after the transfection by Western blots, as described in the Materials & Methods section. Note a decrease but not complete abrogation of P53 expression in the siRNA-transfected cells.

Figure 2. siRNA-mediated inhibition of P53 gene expression causes cell death in HSB-2 cells. The vector for p53-specific siRNA or control vector was transfected in HSB-2 cells. The cells transfected with the p53-specific siRNA vector underwent cell death. The Figure shows cell death in the siRNA and control vector-transfected HSB-2 cells 24 (A) and 48 hours (B) after the transfection, as measured by the Trypan blue exclusion assay. The Figure shows results (mean and SD) from three independent experiments. The difference in means was significant ($p<0.05$) in both A and B.

Figure 3. Effects of a pan-caspase inhibitor, Rapamycin and 3-MA on the cell death mediated by p53-specific siRNA. The cells were transfected with p53-specific siRNA or control vector, and incubated at 37˚C in humidified 5% CO_2 atmosphere in the presence of vehicle (DMSO), Rapamycin (1ug/ml), Z-VAD-fmk (10 ug/ml) or 3-MA (100 uM). Cell death was determined by Trypan blue exclusion 24 (A) and 48 hours (B) after the transfection. The Figure shows cell death (mean and SD) from three independent experiments. Only addition of 3-MA significantly ($p<0.05$) reduced cell death in the siRNA-transfected cells compared with the control vector-transfected cells.

Figure 4. Inhibition of P53 expression with siRNA induces autophagy in HSB-2. The cells were transfected with P53-specif siRNA or control vector, and were incubated in the presence of vehicle (DMSO), Rapamycin or 3-MA. After 24 hours' incubation, the cells were stained intracellularly with FITC-conjugated anti-LC-3 and DAPI. The stained cells were examined under a confocal microscope. A. Photomicrographs of the cells. B. Percentages of autophagic cells. Rapamycin significantly increased ($p<0.05$) autophagic cells in the control vector-transfected cells, whereas 3-MA significantly ($p<0.05$) reduced their number in the siRNA vector transfected cells.

Figure 5. Effect of a pharmacological antagonist of p53, PFT, on cell death.
A. HSB-2 cells were incubated at 37˚C in humidified 5% CO_2 atmosphere in the presence of vehicle (DMSO) or PFT (30 uM) in the presence or absence of 3-MA, or the caspase inhibitor. Cell death was determined after 24 hours' incubation. B. The experiment was repeated using Nutlin-3a (10 mM), in place of PFT. The Figure shows results from three independent experiments.

Figure 6. Inhibition of P53 expression in a p53 mutant human T cell line. CEM were transfected with P53-specific siRNA or control vector, and the transfected cells were incubated at 37˚C in humidified 5% CO_2 atmosphere in the presence of vehicle (DMSO), Rapamycin or 3-MA. After 24 hours, cell death was detrermined by Trypan blue exclusion assay. The Figure shows results (mean and SD) from three independent experiments.

Figure 7. The cell death induced by Pifithrin or Nutlin-3 is not typical apoptosis. In order to determine whether the cell death induced by Pifithrin-α Nutlin-3 apoptotic features, we stained cells with FITC-conjugated Annexin-V and PI. The stained cells were examined by flow cytometry. Note that the treated cells become permeable to PI but do not stain with FITC-Annexin V.

Figure 8. Effects of NAC on PFT-α-mediated cell death.
For this purpose, HSB-2 cells were cultured in the presence of vehicle (DMSO), PFT-α with without the addition of NAC. After 24 (A) and 48 hours (B), cell death was examined by Trypan blue exclusion assay. The Figure shows results (mean and SD) from three independent experiments. No significant differences were observed between PFT- α-treated cells in the presence and absence of NAC ($p>0.05$).

Figure 9. Effects of NAC on Nutline-3-mediated cell death.
The experiment described in Figure 8 was repeated replacing PFT with Nutlin-3. After 24 (A) and 48 (B) hours, cell death was examined by Trypan blue exclusion assay. The Figure shows results (mean and SD) from three independent experiments. No significant differences were observed between Nutlin-3-treated cells in the presence and absence of NAC ($p>0.05$).

Figure 10. PFT induces cell death in primay human T cells.
Primary human T cells, isolated from peripheral blood from healthy donors, were cultured in the culture medium containing 10 units per ml of human recombinant IL-2 in the presence of vehicle (DMSO), PFT or Nutlin-3a. Cell death was determined 24 (A) and 48 (B) hours later. The Figure shows results from a typical experiment. The dead cell numbers were significantly higher ($p<0.05$) in the Nutlin-3 and PFT-treated microcultures than the vehicle-treated cells both 24 and 48 hours later.

Figure 11. The effect of Necrostatin in PFT- and Nutlin-3a-mediated cell death. HSB-2 cells were treated with Nutlin-3 or with PFT in the presence or absence of Necrostatin-1 (100 uM). Cell death was counted 24 hours (A) and 48 hours later (B). The addition of Necrostatin-1 caused a significant decrease ($p<0.05$) in cell death induced by PFT and Nutlin-3a at both time points.

Figure 12. P53 transcriptional activity is required for HHV-6 replication.
In order to determine the effect of P53 inactivation on HHV-6 replication, we infected HSB-2 cells with HHV-6 and cultured them in the presence of vehicle (DMSO), Pifithrin or Bafilomycin-A. Three days post-infection, we determine the expression of an early viral antigen by using a monoclonal antibody (9A5D12) specific for an early HHV-6 antigen (U27-encoded p41) and an FITC-conjugated secondary antibody. The stained cells were examined by flow cytometry. The Figure shows results of a typical experiment. Note decreased expression of the early viral antigen in PFT-treated HHV-6-infected cells as compared with the vehicle-treated cells. Also note the increased expression in the Bafilomycin-A-treated cells.

Figures

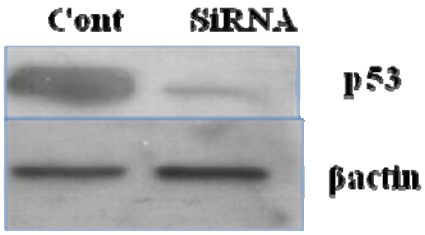
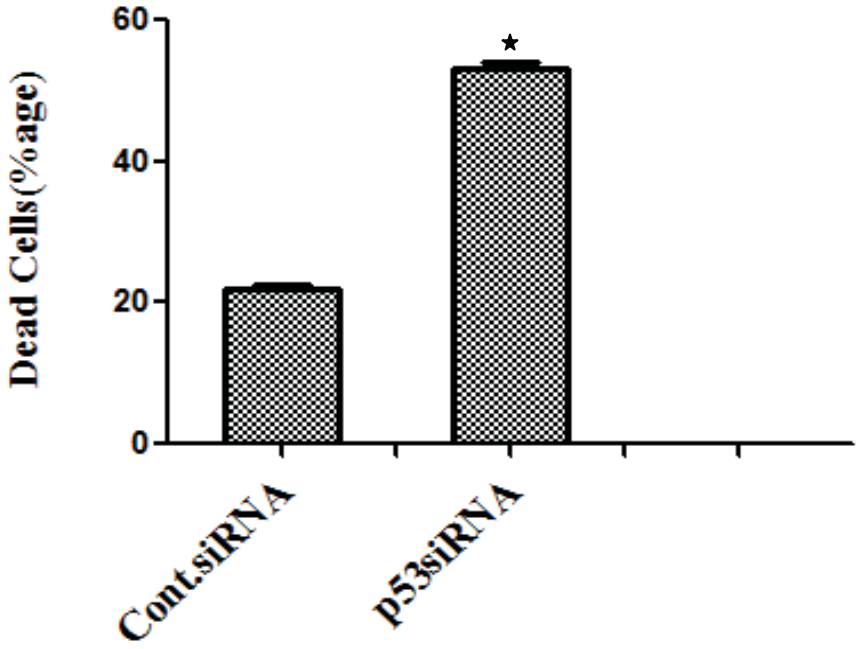


Figure 1

A


60
40
20
0
Dead Cells(%age)
Cont.siRNA
p53siRNA

B

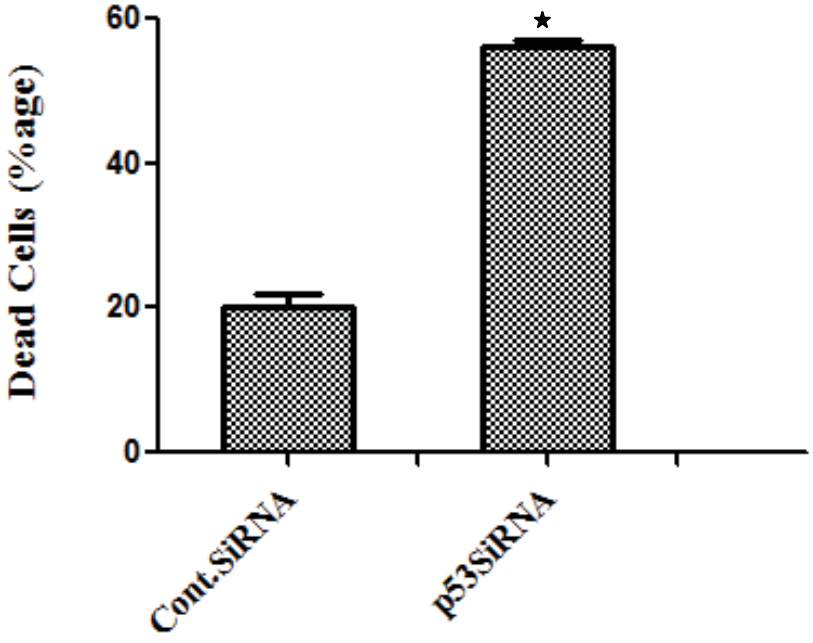
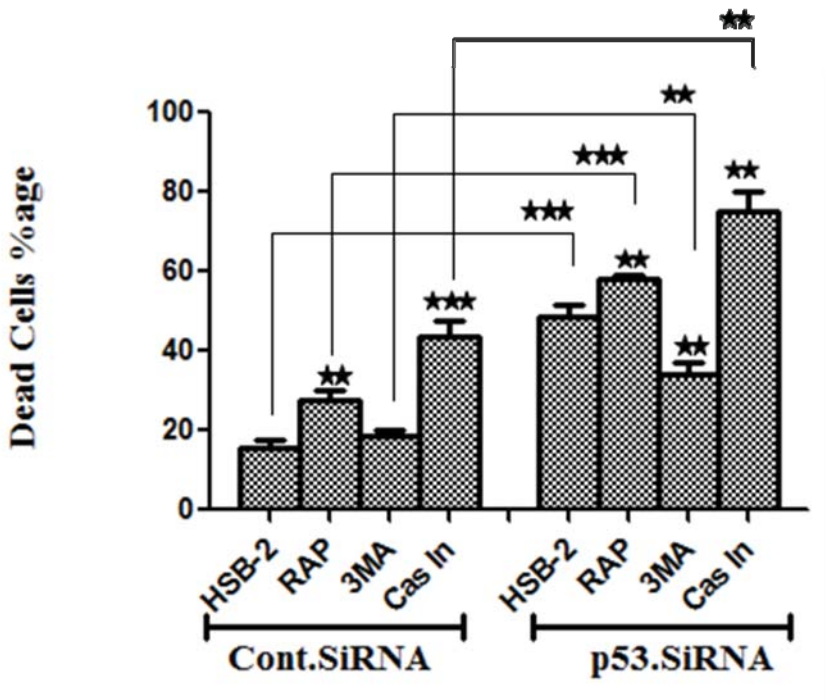

60
40
20
0
Dead Cells (%age)
Cont.SiRNA
p53SiRNA

Figure 2

A


Dead Cells %age
100
80
60
40
20
0
HSB-2
RAP
3MA
Cas In
HSB-2
RAP
3MA
Cas In
Cont.SiRNA
p53.SiRNA

B

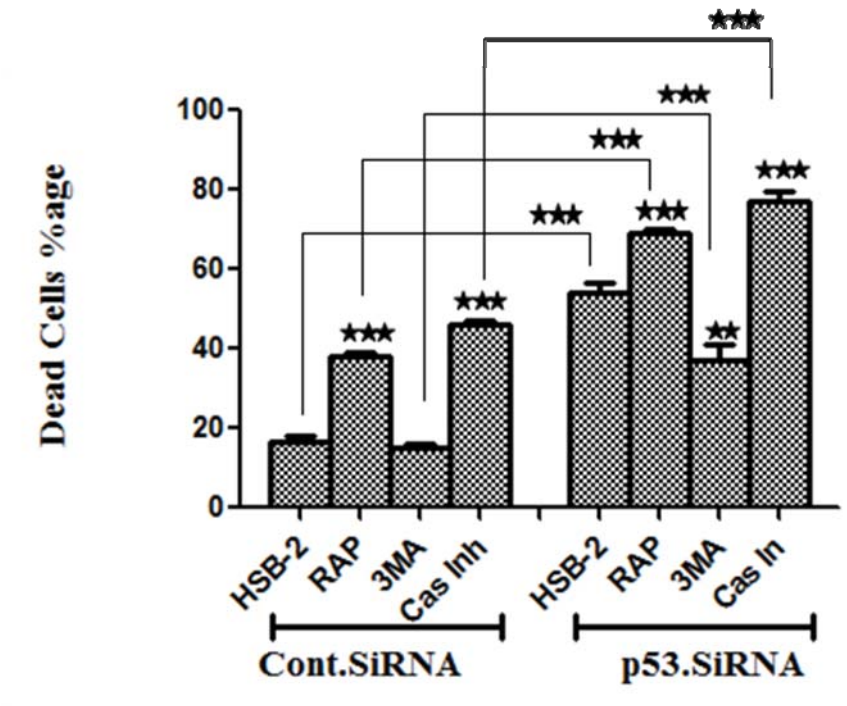

Dead Cells %age
100
80
60
40
20
0
HSB-2
RAP
3MA
Cas Inh
HSB-2
RAP
3MA
Cas In
Cont.SiRNA
p53.SiRNA

Figure 3

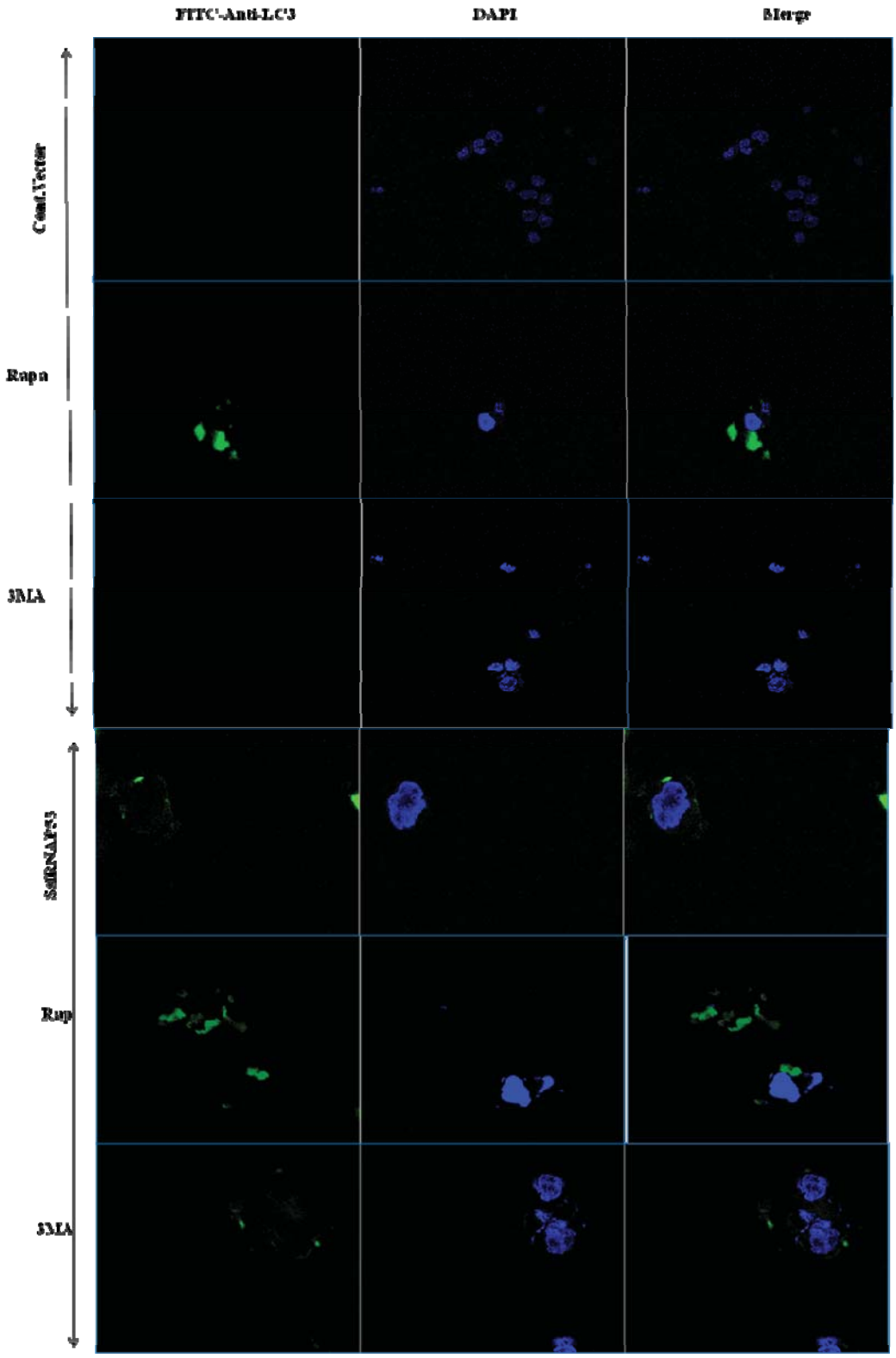

FITC-Anti-LC3
DAPI
Merge
Cont.Vector
Rapa
3MA
SiRNA-P53
Rapa
3MA

Figure 4A

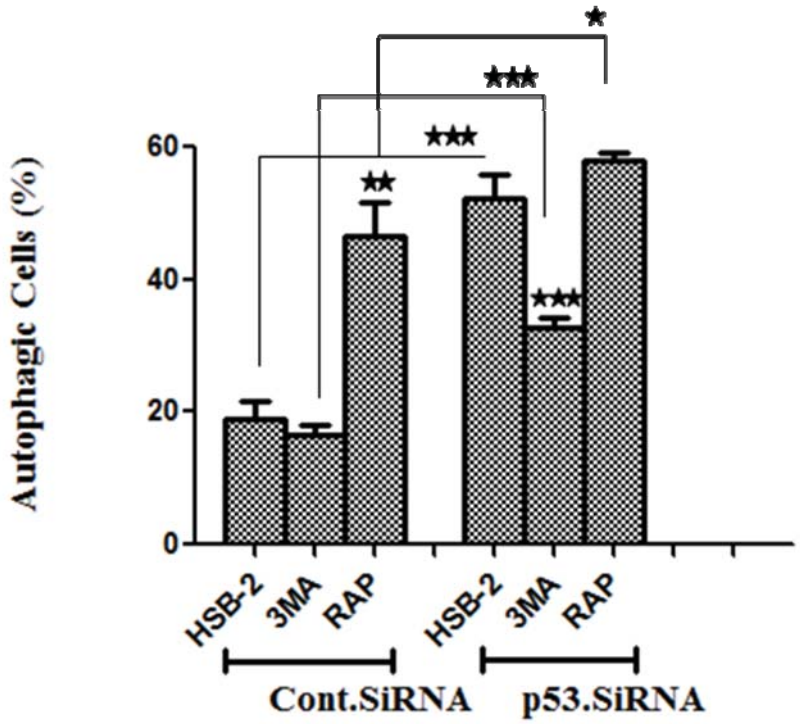
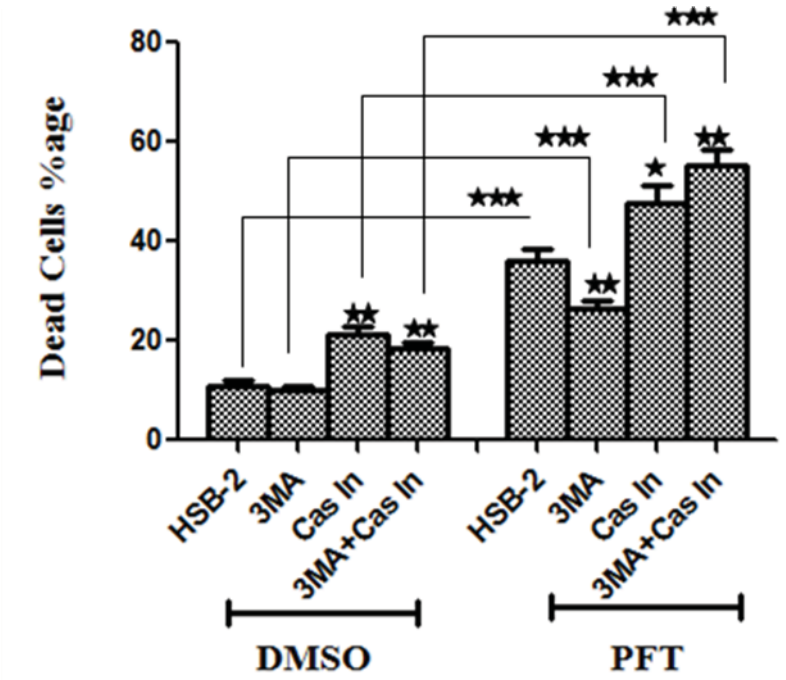

Autophagic Cells (%)
60
40
20
0
HSB-2
3MA
RAP
HSB-2
3MA
RAP
Cont.SiRNA
p53.SiRNA

Figure 4B

A


Dead Cells %age
80
60
40
20
0
HSB-2
3MA
Cas In
3MA+Cas In
HSB-2
3MA
Cas In
3MA+Cas In
DMSO
PFT

B

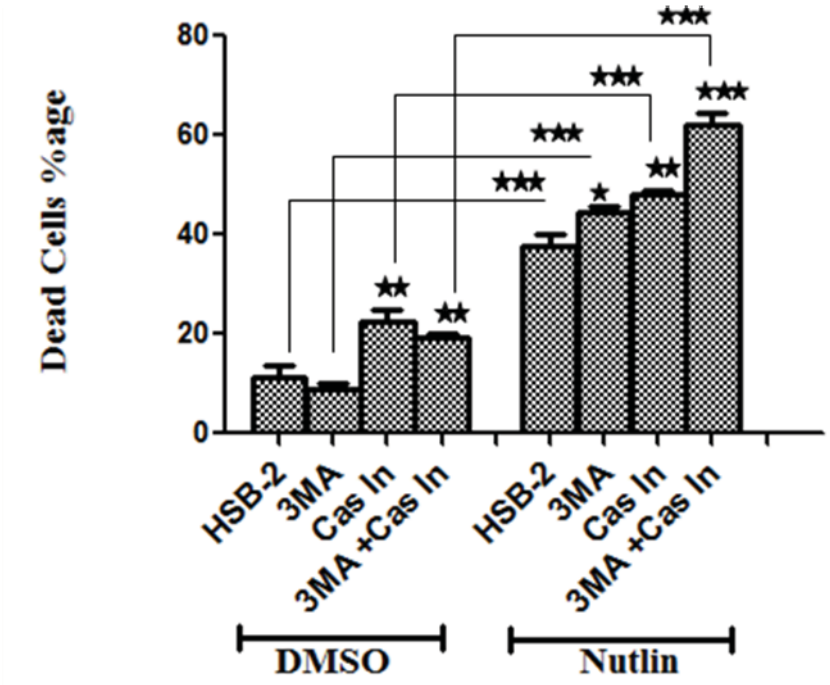

Dead Cells %age
80
60
40
20
0
HSB-2
3MA
Cas In
3MA +Cas In
HSB-2
3MA
Cas In
3MA +Cas In
DMSO
Nutlin

Figure 5

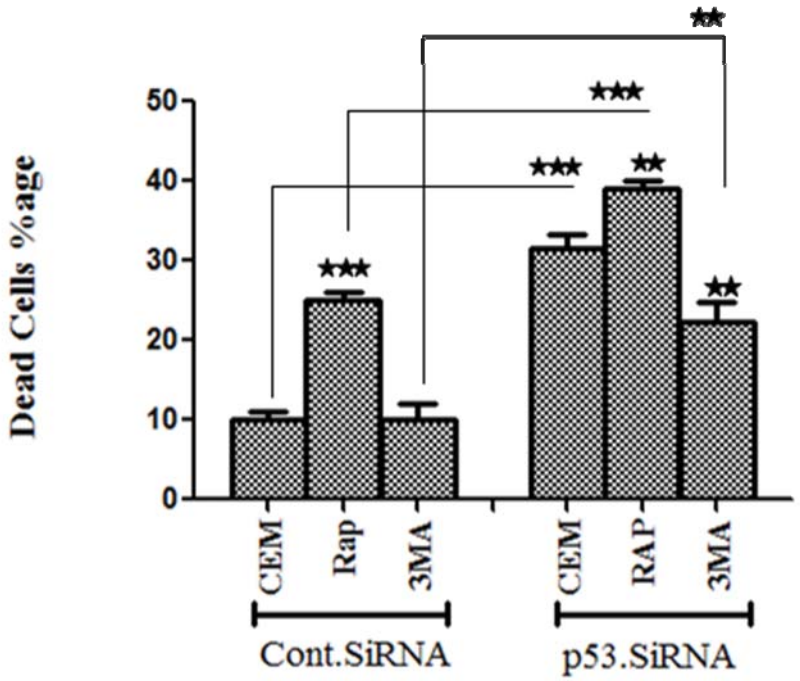
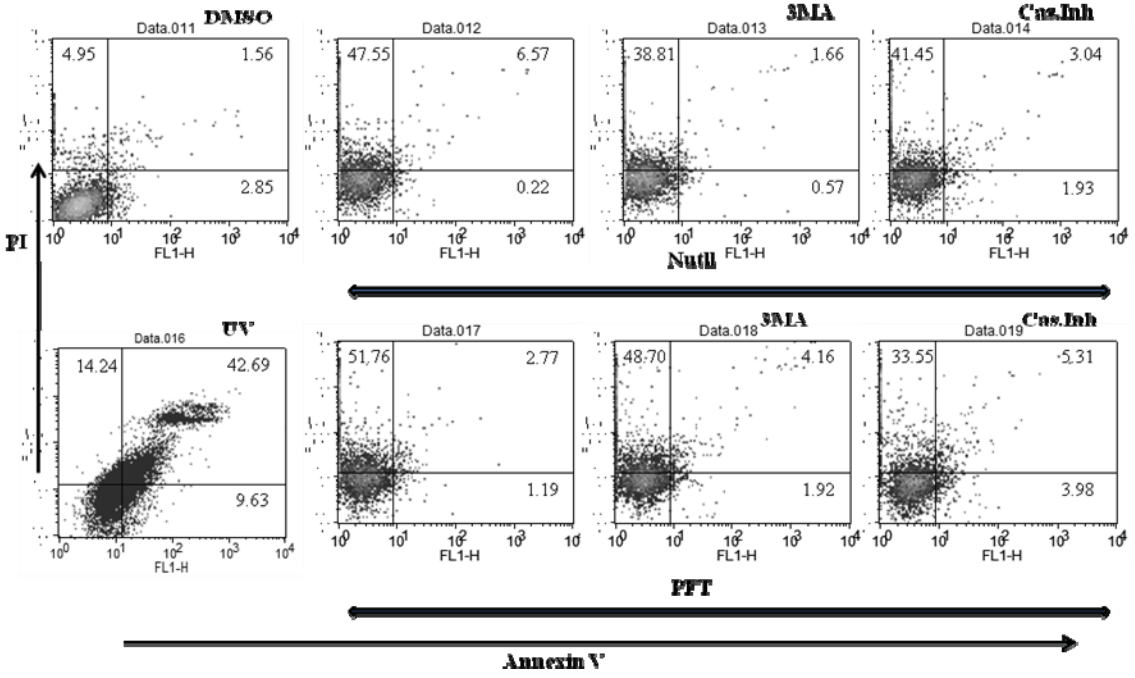

Dead Cells %age
50
40
30
20
10
0
***
***
**
***
**
CEM
Rap
3MA
CEM
RAP
3MA
Cont.SiRNA
p53.SiRNA

Figure 6


DMSO
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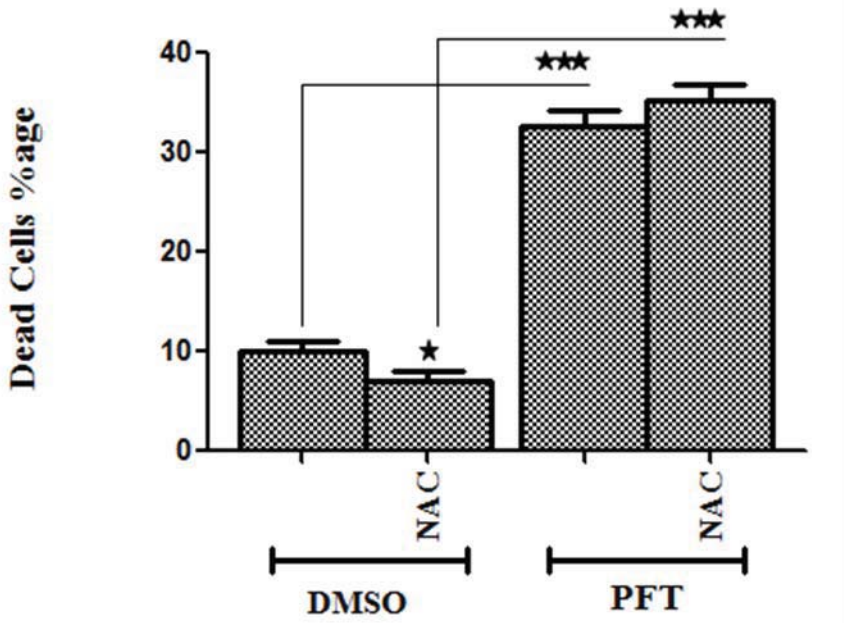
4.95
1.56
2.85
Data.012
47.55
6.57
0.22
3MA
Data.013
38.81
1.66
0.57
C'ns.Inh
Data.014
41.45
3.04
1.93
PI
FL1-H
Nutll
UV
Data.016
14.24
42.69
9.63
Data.017
51.76
2.77
1.19
3MA
Data.018
48.70
4.16
1.92
C'ns.Inh
Data.019
33.55
5.31
3.98
PFT
Annexin V

Figure 7

A


Dead Cells %age
40
30
20
10
0
★★★
★★★
★
NAC
NAC
DMSO
PFT

B

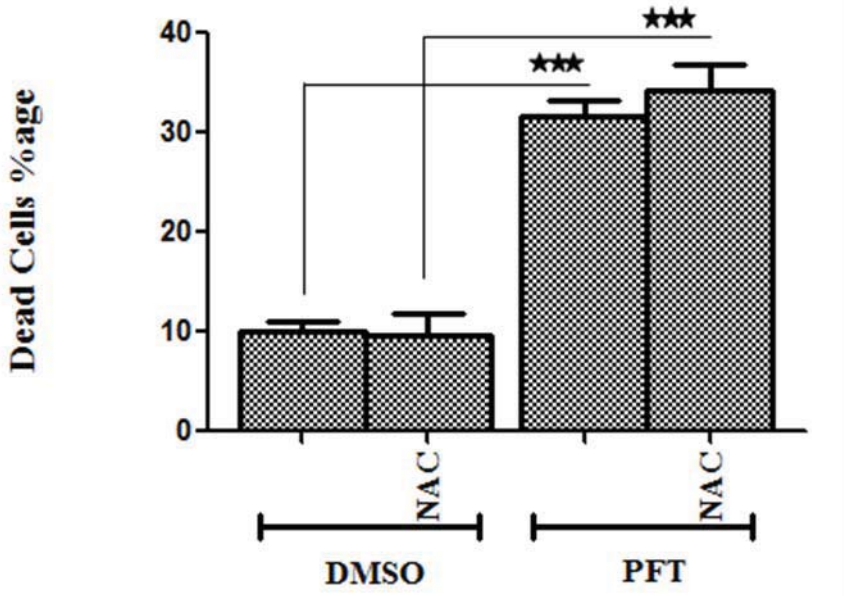
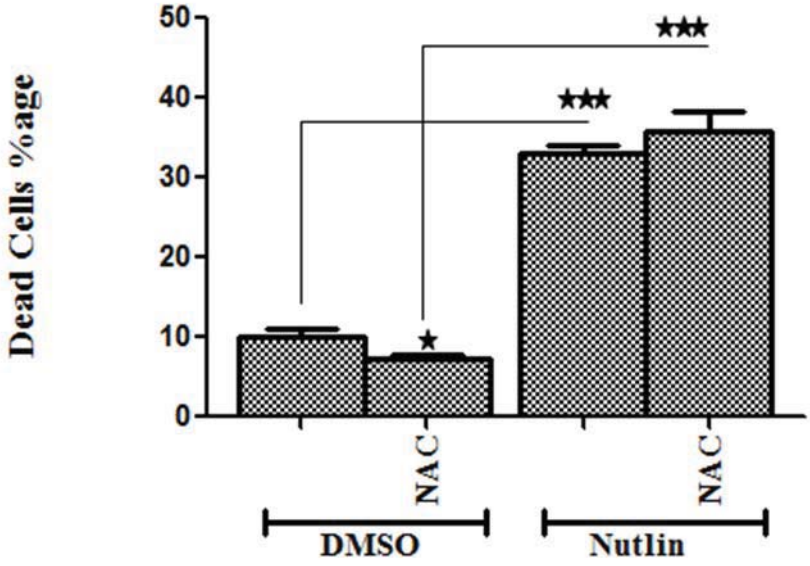

Dead Cells %age
40
30
20
10
0
★★★
★★★
NAC
NAC
DMSO
PFT

Figure 8

A


Dead Cells %age
50
40
30
20
10
0
★★★
★★★
★
NAC
NAC
DMSO
Nutlin

B

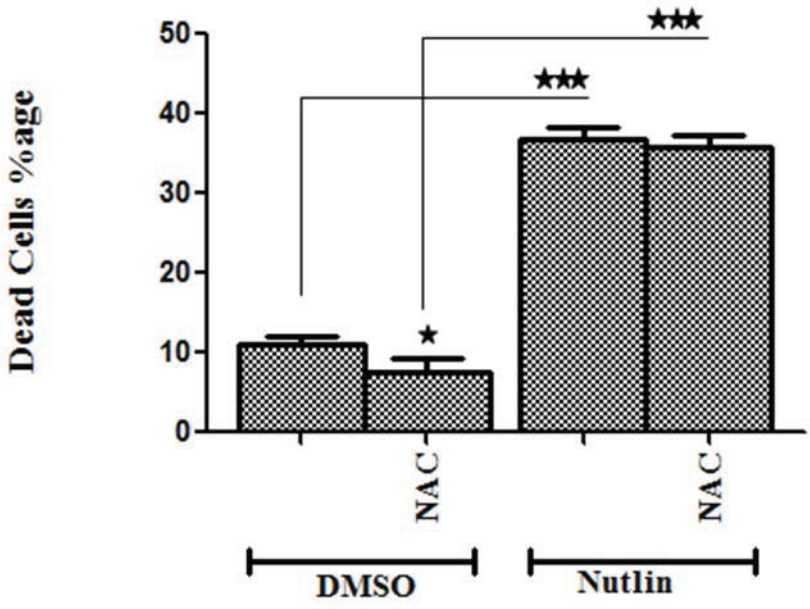
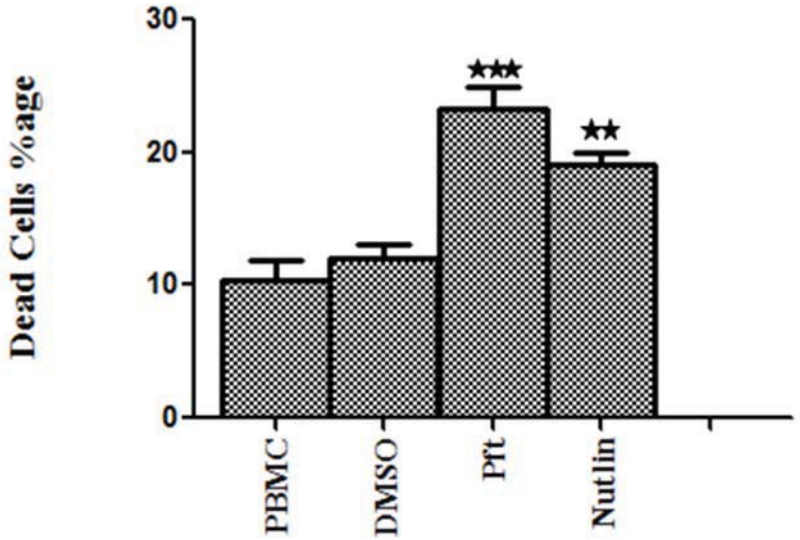

Dead Cells %age
50
40
30
20
10
0
★★★
★★★
★
NAC
NAC
DMSO
Nutlin

Figure 9

A


Dead Cells %age
30
20
10
0
***
**
PBMC
DMSO
Pft
Nutlin

B

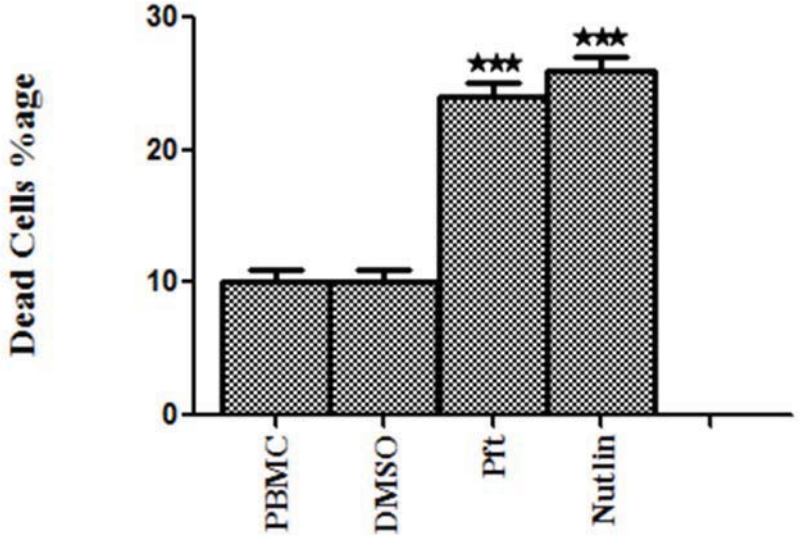
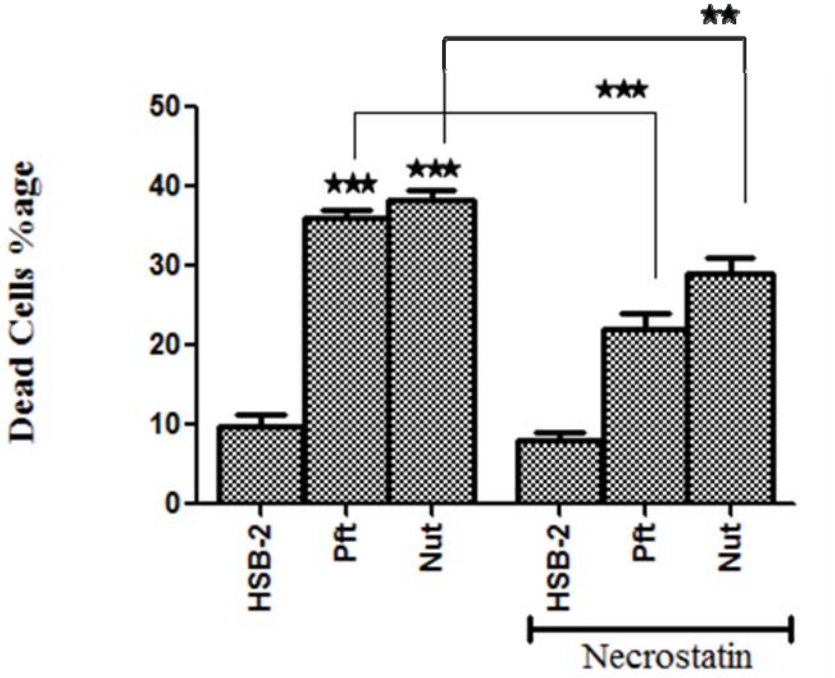

Dead Cells %age
30
20
10
0
***
***
PBMC
DMSO
Pft
Nutlin

Figure 10

A


Dead Cells %age
50
40
30
20
10
0
***
***
**
***
HSB-2
Pft
Nut
HSB-2
Pft
Nut
Necrostatin

B

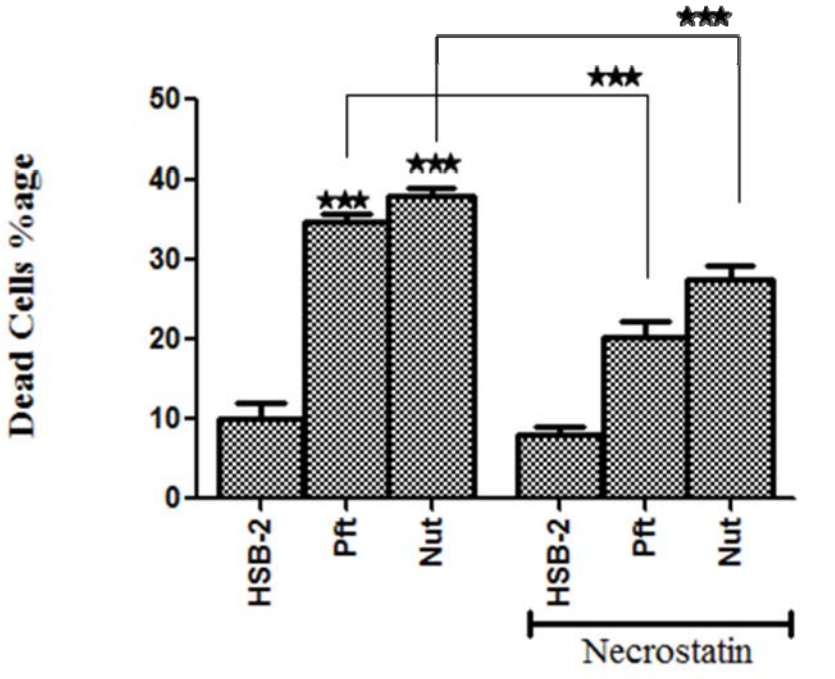

Dead Cells %age
50
40
30
20
10
0
***
***
***
***
HSB-2
Pft
Nut
HSB-2
Pft
Nut
Necrostatin

Figure 11

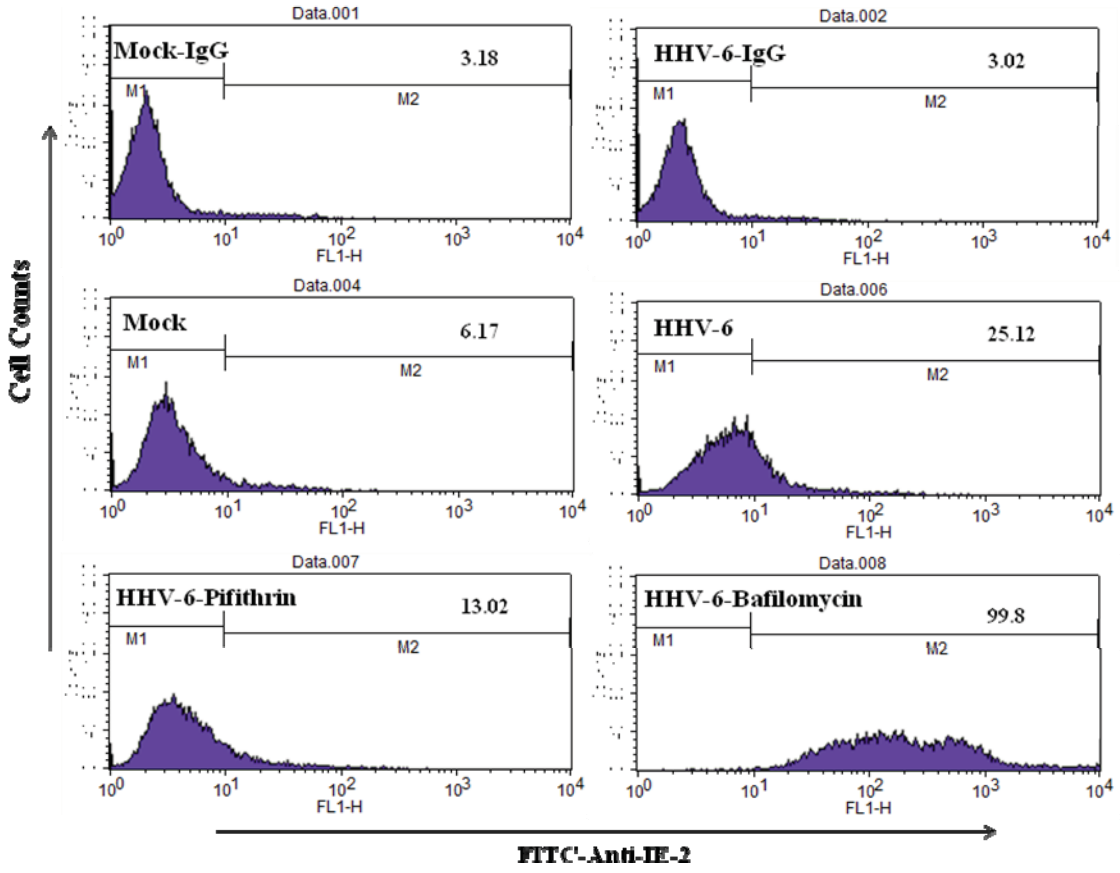

Data.001
Mock-IgG
3.18
M1
M2
Data.002
HHV-6-IgG
3.02
M1
M2
Data.004
Mock
6.17
M1
M2
Data.006
HHV-6
25.12
M1
M2
Data.007
HHV-6-Pifithrin
13.02
M1
M2
Data.008
HHV-6-Bafilomycin
99.8
M1
M2
FL1-H
Cell Counts
FITC-Anti-IE-2

Figure 12

Chapitre 3

Discussion

5.1. HHV-6 et le cycle cellulaire

Le cycle cellulaire représente un ensemble d'événements biologiques bien coordonnés et contrôlés qui donnent lieu à la division d'une cellule en deux cellules filles presque identiques; revue dans (57, 279, 280). Au cours de ce processus, la cellule duplique son ADN et ses diverses organelles, elle augmente son volume cytoplasmique et elle se divise fidèlement entre ses deux cellules filles. La cellule se prépare à la phase G1 et elle assure la disponibilité des enzymes, de l'ensemble des nucléotides et des autres facteurs nécessaires à la synthèse d'ADN. Durant la phase S, la cellule synthétise et duplique effectivement son ADN. Cette phase est suivie par la phase G2, durant laquelle les cellules dupliquent leurs organelles (mitochondries, ribosomes, etc), augmentent leurs volumes cytoplasmiques et doublent quasiment de taille. La phase G2 veille à ce que les cellules filles aient la même taille et les mêmes contenus cytoplasmiques que leur cellule mère. Par conséquent, G2 est la phase la plus active métaboliquement. Sans elle, les divisions cellulaires auraient entraîné une diminution progressive de la taille des cellules. Il a été bien démontré que les virus exploitent différents événements biologiques du cycle cellulaire afin de promouvoir leur propre réplication (40, 85, 206). Ils codent pour différentes protéines qui interagissent avec des facteurs cellulaires pour modifier et réguler la progression du cycle cellulaire. Les informations sur la régulation des machineries du cycle cellulaire par différents virus permettent de mieux comprendre la pathogenèse virale. Ces connaissances pourraient aussi nous aider dans le développement de nouveaux médicaments pour contrôler la réplication virale.

HHV-6 est un pathogène humain ubiquitaire. Ce virus a été associé à de nombreuses pathologies; revue dans (281, 282). Notamment que les infections par HHV-6 chez les nouveau-nés et les réactivations virales chez les personnes immunodéprimées (en particulier les receveurs d'organes et de moelles osseuses) entraînent souvent des troubles médicaux graves. L'infection de ces cellules par HHV-6 est importante et entraine de sérieux problème vu sa capacité à induire des maladies neurologiques. Contrairement aux autres virus, on ignorait presque tout de l'impact de ce virus sur la progression du cycle cellulaire dans les cellules humaines. Ce fut particulièrement le cas lorsque nous avons commencé ce projet. Les quelques études qui avaient abordé cette question ont rapporté des résultats contradictoires. Une étude a rapporté dans ce contexte, que la GS souche de HHV-6 A a des effets suppresseurs sur la prolifération des lymphocytes T humains qui sont induites par les mitogènes et l'IL-2 (283). Les chercheurs ont rapporté l'arrêt des cellules infectées par HHV-6 dans la phase G1 du cycle cellulaire. Ils ont également noté que ces effets viraux sont indépendants de la réplication virale, puisque les virus déficients inactivés par les UV ont été également en mesure d'arrêter les cellules T humaine en G1. Deux études subséquentes ont montré que le HHV-6 infecte *in vitro* les cellules précurseurs gliales en croissance à la fois d'origine humaine et murine, qu'il arrête leur prolifération à la phase G1/S du cycle cellulaire (284, 285).

Contrairement à ces résultats, il a été montré que ce virus entraîne l'arrêt du cycle cellulaire à la phase G2 dans des lymphocytes humains du sang de cordon (234). Ces chercheurs ont montré que ce sont les protéines virales IE synthétisées *de novo* qui induisent cet arrêt du cycle cellulaire.

Les cellules infectées accumulaient p53, CDK1 phosphorylée sur Tyr-15, cycline B et cycline A. L'arrêt se produisait indépendamment de p53 et de ses molécules effectrices p21 et 14-3-3σ. Un autre groupe de chercheurs a rapporté l'arrêt des cellules T humaines infectées par la variante B (PL-1) du HHV-6 dans les phases G1/S et G2/M. Ce groupe a rapporté que cet arrêt dépendait et coïncidait avec la stabilisation de p53 et de sa phosphorylation sur les résidus Ser 15 et Ser 20 (235). Ils ont également noté l'arrêt des cellules épithéliales infectées par le virus en G1/S indépendamment de l'activation de p53, puisque le virus infecte et induit l'arrêt dans les cellules p53-nulles. En outre, ils ont signalé que l'inhibiteur de p53, PFT-α, a été incapable d'inverser cet arrêt (286).

Il faut noter que les équipes qui avaient étudié l'arrêt du cycle cellulaire dans les cellules infectées par HHV-6, avaient utilisé des méthodes basées sur la teneur en ADN dans les cellules. Dans ces méthodes, les cellules sont généralement perméabilisées, traitées à la RNase pour dégrader l'ARN contenue dans les cellules et ensuite elles sont marquées au PI. Ce colorant marque les cellules en fonction de leur teneur en ADN, qui varie quantitativement selon les différentes phases du cycle cellulaire. Les pourcentages des cellules avec différents contenus d'ADN (ou les phases du cycle) sont déterminés par la cytométrie en flux (287). Ces méthodes sont très utiles dans l'analyse de la progression du cycle cellulaire dans les cellules en croissance normale. Elles sont également efficaces pour déterminer les effets des différentes drogues sur le profil du cycle cellulaire des cellules. Toutefois, elles peuvent induire des erreurs dans les résultats lorsqu'elles sont appliquées à des cellules infectées par des virus à ADN. L'ADN viral s'accumule en grande quantité dans les cellules infectées par un virus. La coloration par le PI est également efficace pour le marquage d'ADN viral. Les méthodes de cytométrie en flux ne sont pas en mesure de distinguer entre les ADNs cellulaires et virales. Par conséquent, si les analyses sont basées simplement sur la teneur en ADN, les cellules infectées par le virus qui sont en phase G1 peuvent paraitre en S ou encore en phase G2/M. Ainsi, les analyses du cycle cellulaire des cellules infectées par des virus doivent tenir compte de la présence de l'ADN viral qui y est. Nous avons démontré ici que l'infection de la lignée cellulaire humaine HSB-2 par HHV-6 retarde la progression du cycle cellulaire. Les cellules infectées par ce virus semblent s'accumuler dans les phases S et G2/M du cycle cellulaire. Cependant, lorsque nous avons traité les cellules infectées avec la DNAse micrococcale et avons analysé le cycle cellulaire, nous avons observé une accumulation de ces cellules dans la phase G2/M et non dans la phase S. Le traitement par la DNAse n'a eu aucun effet sur le profil du cycle cellulaire des cellules faussement-infectées. Ces résultats montrent que le traitement n'affecte que le profil du cycle cellulaire des cellules infectées par le virus. Il est connu que l'ADN micrococcale

dégrade l'ADN cellulaire inter-nucléosomale et/ou transcriptionnellement active (288). Étant donné que seule une petite fraction d'ADN cellulaire est transcriptionnellement active durant un certain laps de temps, où elle est sensible à cette DNAse, le traitement des cellules perméabilisées par cette enzyme ne réduit pas assez l'ADN cellulaire au point d'affecter l'analyse du cycle cellulaire. D'autre part, l'ADN des virus herpès n'est pas emballé dans les nucléosomes à l'intérieur des cellules (289), par conséquent, elle est efficacement dégradable par la nucléase micrococcale. Nous expliquons ces résultats de la façon suivante : HHV-6 retarde/arrête les cellules infectées à la phase G2/M. C'est la présence d'ADN viral dans les cellules qui corrompt l'analyse et fait en sorte que les cellules paraissent s'accumuler dans la phase S. En fait, il pourrait s'agir des cellules infectées qui sont en G1 mais qui semblent avoir plus d'ADN et elles apparaissent donc dans la phase S. Des résultats similaires ont été obtenus lorsque nous avons infecté des PMBC humains activés avec du PHA et d'IL-2, ou encore des cellules purifiées T CD4+ primaires. Nos résultats concordent avec ceux décrits précédemment concernant les lymphocytes du sang de cordon infectés par HHV-6 (234). Cependant, dans notre cas, les cellules infectées par ce virus s'accumulent progressivement dans les phases S et G2/M en absence de traitement par la DNase micrococcale.

Toutefois, nous n'avons pas observé de changements remarquables dans le profil du cycle cellulaire des cellules infectées par ce virus, quand elles ont été traitées avec l'acide phosphonoacétique, un inhibiteur de l'ADN polymérase virale. En plus, nous observons les effets du virus sur le cycle cellulaire à partir d'environs 6-7 jours après infection. Ces résultats suggèrent que les changements dans les profils du cycle cellulaire ne sont pas dus à l'expression des protéines virales IE. Ces différences entre nos résultats et ceux de Bolle et al (234) pourraient s'expliquer par l'utilisation de différents types de cellules lors de ces études. Sans traitement par la DNase, nous avons obtenu des résultats globalement similaires à ceux de Dietrich et al., et Mock et al. (284, 285). Nous n'avons jamais observé d'accumulation des cellules infectées par ce virus dans la phase G1, tel qu'il a été décrit par certains chercheurs (235, 283, 286). Encore une fois les différences dans les résultats observés entre leurs études et la notre pourraient s'expliquer par l'utilisation de différents types cellulaires, protocoles de culture cellulaire, préparations virales, doses virales infectantes, etc. Les virus interférent avec la progression du cycle cellulaire dans le but d'exploiter les facteurs des cellules hôtes et des nucléotides pour leur propre réplication. En même temps, ils ont tendance d'empêcher la réplication d'ADN cellulaire.

Par conséquent, tout dépend de leurs besoins, les divers virus peuvent causer différents changements au niveau de la progression du cycle cellulaire dans les cellules infectées. Il a été montré que les infections lytiques des cellules humaines par le HSV-1, le HCMV et l'EBV arrêtent ces cellules dans la phase G1 du cycle cellulaire (40). Néanmoins, HHV-6 diffère de ces virus du fait que son infection productive cause l'arrêt des cellules infectées dans la phase G2/M du cycle cellulaire. Des arguments pourront être soulevés comme le fait que les cellules utilisées dans notre étude pourraient être

intrinsèquement incapable de s'arrêter dans la phase G1 du cycle cellulaire. Nous avons étudié cette possibilité et nous avons constaté que les cellules HSB-2 sont bien capables de subir l'arrêt à la phase G1 du cycle cellulaire suite à un stimulus approprié, par exemple, la dexaméthasone (données non présentées). Fait intéressant, le HHV-7 qui ressemble étroitement à HHV-6 provoque également l'arrêt du cycle cellulaire à la phase G2/M du cycle cellulaire (238).

Pour les cellules humaines infectées par HHV-7, l'activité de la kinase mitotique (le complexe cdc2/cycline B) est significativement diminuée, tandis que l'expression de cdc2 et de cycline B reste élevée (238). Pour ces cellules, le modèle cyclique d'expression habituel de la cycline B est perdu. La protéine sigma 1 du réovirus, sérotype 3 (souche Abney) induit également l'arrêt du cycle cellulaire à la phase G2/M, causée par une diminution de l'expression de la sous-unité régulatrice β de PP2A (Protein Phosphatase-2A) et, par conséquent, par une diminution de la forme hyperphosphorylée et inactive de cdc2 (290, 291). La cycline B est le partenaire catalytique de la kinase mitotique CDK1. L'activité fonctionnelle de cette kinase est régulée par la production et la destruction de la cycline B à la fin de la phase G2. À la fin de la phase M et au début de l'anaphase, cette cycline est dégradée (292, 293).

Nous avons également observé une diminution d'expression de la cycline B dans les cellules infectées par HHV-6. Conformément à ces résultats, l'activité kinase de la cycline B immune précipitée à partir des cellules infectées par ce virus a été significativement réduite par rapport à celle des cellules mock-infectées. La cycline B est l'un des partenaires catalytiques connus de CDK1 (cdc2) et généralement, il n'y a pas de différences visibles entre les activités kinase des cdc2 et celles des cyclines B immunoprécipitées (292). Nous avons observé par contre des activités kinase élevées des cdc2 immunoprécipitées à partir de cellules infectées par HHV-6. D'après nos résultats d'analyse par microarray du profil d'expression des gènes régulateurs du cycle cellulaire (données non publiées), il n'y a pas de diminution d'expression du gène de la cycline B dans les cellules infectées. Cependant, la forte diminution observée dans l'expression de cette cycline semble provenir de sa dégradation protéasomale.

La dégradation protéasomale hautement contrôlée des cyclines et des inhibiteurs des kinases dépendantes des cyclines. Elle joue un rôle essentiel dans la progression normale du cycle cellulaire; revue dans (294, 295).

Les hauts niveaux d'activités fonctionnelles de la kinase mitotique face à des niveaux indétectables de cycline B dans les cellules infectées par HHV-6 soulève des questions sur la nature du partenaire catalytique de CDK1 (cdc2) dans ces cellules. Puisque la cycline A peut également agir comme un partenaire catalytique de cette kinase, nous avons déterminé alors l'expression de cette cycline ainsi que l'activité kinase associée à son complexe immun. Nous n'avons pas observé une diminution dans l'expression de la cycline A dans les cellules infectées par HHV-6 par rapport à celles mock-infectées. Toutefois, nous avons observé une diminution de l'activité kinase des complexes

immuns associés à cycline A dans les cellules infectées par rapport à celles mock-infectées. Ces données suggèrent que l'augmentation observée de l'activité kinase associée à cdc2 dans les cellules infectées pourrait ne pas être due à son association avec cette cycline. L'augmentation de l'activité kinase des cdc2 immunoprécipitées obtenue à partir de cellules infectées par HHV6 a été tout à fait inattendu, puisque nous avons observé une diminution de l'expression de cette kinase dans ces cellules par rapport aux cellules mock-infectées. Il est probable que le virus puisse coder pour une protéine qui agit comme un partenaire pour cdc2 et catalyse son activité kinase. Cette notion est soutenue par une étude qui a rapporté que le DPPF (*DNA polymerase processivity factor*), codé par le gène UL42 de HSV-1 se complexe avec cdc2 et agit comme son partenaire catalytique dans les cellules infectées par HSV (296).

Le DPPF contient une boîte cycline dégénérée et il ressemble structurellement à l'antigène nucléaire de prolifération cellulaire (PCNA), qui lui-même se lie à CDK2 (297). Cdc2 phosphoryle DPPF à un site cryptique près de son extrémité carboxy-terminale. En outre, DPPF immunoprécipité à partir des cellules infectées par HSV-1- montre une activité kinase similaire à celle de cdc2 lors des mesures de kinase *in vitro*. Nous avons entrepris une recherche informatique qui a montré que HHV-6 code également à un homologue de DPPF, doté d'un site potentiel de phosphorylation pour cdc2 (données non publiées). Nous croyons qu'il pourrait former un complexe avec cdc2 et catalyser son activité kinase en dépit de la dégradation de la cycline B dans les cellules infectées par HHV-6.

Le maintien d'un niveau élevé de l'activité de cdc2 dans les cellules humaines infectées par HHV-6 suggère que cette kinase pourrait jouer un rôle important dans la biologie de HHV-6. Le site de phosphorylation canonique de cette kinase est connu pour être S/T-P-X-K/H/R (298). Il est peut être pertinent de mentionner que 27 orf de HSV-1 peuvent coder pour des protéines avec des sites de phosphorylation pour cette kinase et, par conséquent, peuvent potentiellement être phosphorylés par cdc2 (299). De plus, nous avons entrepris une recherche informatique qui a montré qu'il y aurait au moins 34 protéines codées par HHV-6 qui peuvent avoir ce motif (données non présentées). Une ou plusieurs de ces protéines pourraient être potentiellement ciblées et phosphorylées par cette kinase et pourraient être importantes pour la réplication virale. Dans ce contexte, il a été démontré que la phosphorylation des protéines de HSV-1 médiée par cdc2 est essentielle à la réplication virale. Les cellules exprimant un mutant dominant négatif de cdc2 infectées par HSV-1, n'arrivent pas à exprimer un sous-groupe de protéines virales tardives (299). En outre, la roscovitine et l'olomoucine, deux inhibiteurs spécifiques des plusieurs CDK notamment de cdc2, inhibent la réplication de HSV-1 *in vitro* (300, 301). Comme mentionné ci-dessus, nous avons trouvé 34 orf qui pourraient coder pour des protéines avec un motif canonique pour cette kinase. Nous émettons l'hypothèse suivante; la phosphorylation d'un ou de plusieurs de ces protéines est essentielle à la réplication de HHV-6 dans les cellules humaines. De toute évidence, des travaux supplémentaires sont

requis pour répondre à ces questions. Cdc2 ou son partenaire putatif viral pourrait servir de molécules cibles pour le développement de médicaments anti-HHV-6. Il est à noter que l'activité de cdc2 dans les cellules humaines infectées par HSV-1 est presque la même que celle dans les cellules mock-infectées. Toutefois, elle est beaucoup plus élevée dans les cellules infectées par HHV-6 comparativement à cellules mock-infectées (Table 4 dans le premier papier dans la section « Résultats »). Ceci suggère que les inhibiteurs de cdc2 pourraient avoir un index thérapeutique plus large pour les infections par HHV-6 par rapport à celles par HSV-1.

Il a été montré que le gène U27 de HHV-6, qui est l'homologue de pUL42 de HSV-1, est transcrit avec les gènes précoces- tardifs et code pour une protéine de 41 kD (p41; (302-304)). Nous avons montré, à l'aide des anticorps anti-P41et anti-cdc2, que cette protéine virale co-immunoprécipite avec cdc2 dans les cellules infectées par HHV-6 et vice-versa. En outre, les protéines P41 immunoprécipitées montrent des activités cdc2-like kinase *in vitro* dans des mesures de kinase. Toutes ces données suggèrent que le produit du gène U27 de HHV-6 est le partenaire de cdc2 et qu'il augmente son activité kinase dans les cellules infectées par ce virus. Il est à noter que, même les cellules humaines infectées par HSV-1 ont également une augmentation de l'activité kinase associée à cdc2 malgré la disparition des cyclines A et B (296). Toutefois, les deux virus agissent différemment sur la progression du cycle cellulaire dans les cellules humaines: HSV-1 arrête les cellules dans la phase G/S, tandis que HHV-6 entraine leur accumulation en G2/M. Les différences entre les deux virus au niveau des effets d'autres protéines virales peuvent être responsables de ces effets différentiels sur l'expression du cycle cellulaire

Nos données obtenues par Western blot montrent que l'expression de p53 augmente dans les cellules infectées par HHV-6. Nous avons constaté par contre que les niveaux de la protéine phosphorylée sur Ser15 ont diminué dans ces mêmes cellules. P53 est un gène suppresseur de tumeur qui joue le rôle de chef d'orchestre pour réparer l'ADN s'il y a endommagement, arrêter le cycle cellulaire, induire l'apoptose et la différenciation; revue dans (39, 305-308). P53 exerce également plusieurs effets et elle est impliquée dans différentes voies cellulaires métaboliques (309, 310). Environ la moitié de toutes les tumeurs humaines sont mutantes pour ce gène, tandis que d'autres, peuvent être mutantes pour les gènes qui activent et/ou médient les réponses de p53. Dans les cellules qui se répliquent normalement, la protéine p53 se lie à la protéine MDM2 (*mouse double minute*) et elle est rapidement dégradée par voie protéasomale. Par conséquent, sa demi-vie est très courte dans ces cellules (généralement de quelques minutes). Elle est phosphorylée en réponse aux dommages d'ADN ou suite à certaines infections virales, à des résidus multiples par des différentes kinases qui comprennent le *checkpoint kinase-1* (CHK-1), le *checkpoint kinase-2* (CHK-2), la protéine kinase dépendante d'ADN (DNA-PK) etc (161, 306, 307, 311). Quand p53 est activée, elle ne se lie pas à la protéine MDM2, elle est protégée de la dégradation et elle s'accumule dans les cellules. Nos

données montrent que HHV-6 induit une augmentation des niveaux de la protéine p53 activée. Conformément à ces résultats nous avons constaté que HHV-6 induit l'expression accrue de CHK-1 et CHK-2 dans les cellules infectées par rapport aux cellules mock-infectées. La principale fonction de p53 dans les cellules est d'agir comme un facteur de transcription qui induit l'expression de plusieurs gènes cibles en se liant à l'ADN dans les promoteurs de ces gènes d'une manière spécifique aux séquences. Les gènes cibles comprennent p21, GADD45, Bax, AIP1 (*apoptosis inducing protein-1*) et MDM2 (128, 136, 309, 312, 313).

Comme les cellules HSB-2 disposent d'un gène p53 fonctionnel (314), on s'attendrait à voir une augmentation dans l'expression des gènes induits par p53 ainsi que leurs produits dans les cellules infectées par HHV-6. Cependant, nous avions à peine détecté p21 par Western blot dans les cellules infectées par HHV-6 (voir aussi ci-dessous). Les niveaux de cette CDKI sont beaucoup plus faibles dans les cellules infectées par ce virus comparativement à celles mock-infectées. Ces résultats suggèrent que la protéine p53 pourrait être transcriptionnellement inactive dans les cellules infectées par HHV-6. Cette hypothèse est soutenue par l'étude qui stipule que l'orf 1 (un gène viral IE) de HHV-6 se lie à p53 *in vitro* et inhibe son activation transcriptionnelle potentielle (315, 316); revue en (317). Ces résultats suggèrent également que HHV-6 active p53 dans les cellules infectées, quoiqu'il semble modifier ses fonctions de transcription normales. P21, un membre de la famille cip des inhibiteurs de CDK, c'est le principal médiateur de l'arrêt du cycle cellulaire induit par p53 dans la phase G1 (279, 306, 318). P21 se lie et inactive les cdk des phases G1 et S et induit l'arrêt des cellules en croissance à la phase G1 du cycle cellulaire. Comme il est mentionné ci-dessus, nous n'avons observé aucune augmentation de l'expression de la protéine p21 dans les cellules infectées par HHV-6 par rapport à celles mock-infectées. Au contraire, l'expression de p21 a été diminuée et elle est devenue indétectable dans les cellules infectées par HHV-6. Nous avons observé par Western blot qu'une brève incubation des cellules infectées et mock-infectées dans un milieu de culture contenant un inhibiteur du protéasome restaure l'expression de p21 dans les cellules infectées. Cependant, son niveau d'expression, reste inférieur à celui dans les cellules HSB-2 mock-infectées et traitées similairement. Ces observations suggèrent que p21est synthétisée à des taux moindres et qu'elle est également préférentiellement dégradée dans les cellules infectées par HHV-6 par voie protéasomale. Comme cet inhibiteur est le principal médiateur de l'arrêt du cycle cellulaire induit par p53 dans la phase G1 (306, 318), sa disparition dans les cellules infectées par HHV-6 pourrait expliquer pourquoi ces cellules ne subissent pas d'arrêt en G1. Une diminution d'expression des cdk de la phase G1 dans les cellules infectées soutient également le fait que ces cellules ne s'arrêtent pas dans la phase G1. En dégradant p21, le virus désactive un mécanisme effecteur important par lequel p53 induit l'arrêt du cycle cellulaire en réponse à des stimuli d'endommageant d'ADN.

Dans ces circonstances, les cellules infectées par HHV-6 pourraient devenir instables génétiquement et accumuler l'ADN endommagée.
Des études récentes ont montré que l'activation de p53 entraîne également l'activation de la caspase-2, bien que le mécanisme exact de cette activation reste insaisissable (319, 320).
L'une des cibles de la caspase-2 est p21. La caspase- 2 induite par l'activation de p53 clive et inactive p21 et fait pencher la balance d'arrêt du cycle cellulaire vers l'apoptose (319). Plusieurs études ont montré que les deux variantes A et B de HHV-6 induisent l'apoptose dans les cellules T humaines, particulièrement dans les cellules effectrices mémoires (321-324). P53 et Fas/Fas L ont été impliqué dans l'apoptose induite par HHV-6 (286, 322, 323). Cependant, il a été montré que ce virus induit ce type de mort que dans les cellules bystander non-infectées (237). Il serait intéressant de voir si la caspase-2 joue un rôle dans ces cellules apoptotiques. La diminution d'expression de la p21 en dépit d'activation de p53 dans les cellules infectées par HHV-6, pourrait découler de l'activation de la caspase-2 induite par p53. Des travaux supplémentaires sont nécessaires pour confirmer que l'activation de p53 induite par ce virus mène réellement à l'activation des caspases et au clivage du p21.
Des anomalies dans la progression du cycle cellulaire, en particulier en anaphase et à la cytocinèse, conduisent à l'aneuploïdie. En utilisant le FISH *in situ* et à l'aide de sondes spécifiques à pRb, nous avons détecté une augmentation de la polyploïdie dans les cellules infectées par HHV-6. Ces résultats suggèrent que les cellules infectées par HHV-6 font plus d'erreurs lors de la progression du cycle cellulaire conduisant à une instabilité génomique et à une polyploïdie. Des études supplémentaires seraient nécessaires pour comprendre la nature exacte de ces erreurs.

5.2. P53 et la survie cellulaire

Jusqu'à date, la lignée cellulaire la plus permissive et adaptée à l'infection et à la propagation du virus HHV-6 reste CCRF-HSB-2 (ATCC CCL 120.1), une lignée de cellules T lymphoblastique et leucémiques. Cette lignée dérive du sang périphérique d'un jeune patient Caucasien de 11 ans atteint d'une leucémie lymphoblastique aiguë. Ces cellules ont été reproduites huit fois chez des nouveau-nés des hamsters syriens (325). Ce sont des cellules diploïdes (2n) dotées de p53 de type sauvage quoique leur pRb soit mutée (326). Ces cellules répondent bien aux dommages d'ADN causés par l'irradiation gamma, la cisplatine ou encore le traitement à l'étoposide et ce en activant p53, induisant l'expression de p21 puis en subissant l'arrêt en G1 et par conséquence l'apoptose. Les HSB-2 poussent en suspension (leur temps de doublement est de 36,2 heures), elles sont très sensibles aux variations des facteurs de croissance et de

pH. Il est difficile de les maintenir en culture cellulaire puisqu'il s'agit d'une lignée très radiosensibles et qui subit l'apoptose très rapidement (314).

Malgré que HSB-2 soit la lignée cellulaire la plus permissive pour la réplication de HHV-6, le virus prend un temps relativement long (10-12 jours) pour compléter sa réplication et induire les effets cytopathiques.
Cela, complique la réalisation de nombreuses expériences. Par exemple, la synchronisation du cycle cellulaire devient pratiquement inutile lorsqu'on étudie les effets du virus sur le cycle cellulaire dans cette lignée. En outre, il faut suivre l'infection pendant 12 jours pour étudier la cinétique de la réplication virale pour une fonction cellulaire donnée ou l'expression d'une protéine cellulaires ou virale donnée. Comme mentionné plus haut, cette lignée cellulaire exprime p53 de type sauvage, elle est très radiosensible et subit l'apoptose très rapidement. En outre, les virus sont connus d'induire la réponse aux dommages d'ADN dans des cellules hôtes. Ce qui conduit presque inévitablement à la stabilisation et à l'activation de p53 entraînant l'arrêt du cycle cellulaire et/ ou l'apoptose. Afin de prévenir la mort cellulaire prématurée et l'arrêt du cycle cellulaire dans une phase défavorable, les virus ont développé de nombreuses stratégies pour lier et inactiver p53 dans les cellules hôtes (119, 327). Les petits virus à ADN codent pour différentes protéines virales qui inactivent p53, comme, l'antigène grand T de SV-40, l'E7 de plusieurs souches cancéreuses du virus du papillome humain, etc (119, 327, 328). Pareillement et pour aboutir aux même buts, les herpèsvirus codent pour des protéines virales qui jouent ce rôle.
HHV-6 produit deux protéines virales qui lient p53 (315, 316). La protéine s'accumule dans les cellules infectées où elle est phosphorylée sur Ser 15 (286, 322). D'après l'induction de p53, il semblerait qu'en quelque sorte les cellules infectées ne soient pas favorables à la réplication virale. Par conséquent, le virus doit lutter aux effets de l'activation de p53 en codant pour des protéines liants cette dernière. Dans ce contexte, nous avons émis l'hypothèse suivante : La suppression de l'expression du gène p53 par Knock down dans les cellules HSB-2 pourrait accélérer le rythme de réplication de HHV-6, raccourcir son cycle réplicatif qui est de 10 à 12 jours et pourrait en même temps rallonger la survie de ces cellules en cultures. Par conséquent, nous avons transfecté ces cellules avec des vecteurs qui codent soit pour p53-spécifiques soit pour siARN contrôle. Après 24 heures de transfection, nous avons infecté les cellules par HHV-6 ou par un mock-virus.
Nous avons été surpris de constater que les cellules transfectées avec le vecteur siARN p53-spécifique ont montré une étendue mort cellulaire. Cette mort cellulaire est survenue dans ces cellules, indépendamment de l'infection par le virus. Nous avons obtenu des résultats similaires lorsque nous avons traité les cellules HSB-2 avec un inhibiteur pharmacologique qui est, la Pifithrine-α (PFT). Cet inhibiteur bloque l'activité transcriptionnelle de P53, bien que son mécanisme d'action reste encore inconnu (329). Fait intéressant, l'incubation des cellules avec un antagoniste pharmacologique de p53, la Nutline-3, aboutit également aux

mêmes résultats. Il est à noter que la Nutline-3 inhibe l'interaction entre p53 et MDM-2. Cette dernière est une ligase E3 p53-spécifiques qui induit l'ubiquitination et la dégradation protéasomale de p53. En inhibant la dégradation de p53, Nutline-3 induit son activation et sa stabilisation (330, 331); revue en (332). Ces expériences démontrent qu'aussi bien l'appauvrissement en p53 que sa stabilisation induit la mort cellulaire dans les cellules humaines.

Nous avons répété ces expériences avec une lignée cellulaire T humaine, la CCRF-CEM ayant le gène p53 muté (333). CCRF-CEM est également une lignée de cellules T établie à partir du sang périphérique d'un patient atteint de leucémie lymphoblastique aiguë (CCRF-CEM, Catalogue # CCL-119, ATCC). Des résultats similaires ont été obtenus avec des blastes PHA primaire humain. Les résultats obtenus à partir de différents types cellulaires suggèrent qu'aussi bien l'activation que la déplétion de p53 induisent la mort cellulaire, peu importe si p53 est de type sauvage ou mutant. Nous avons effectué plusieurs expériences pour montrer que la mort cellulaire observée n'était pas de l'apoptose puisque les cellules ne se colorent pas avec l'annexine V-FITC et que cette mort n'a pas été inhibée par un inhibiteur pan-caspase (Z-VAD-FMK; Catalog # 627610; Calbiochem). Il est à noter que plusieurs caspases (caspase 2, 3, 8 et 9) sont essentielles pour l'exécution de toutes les manifestations d'apoptose dans les cellules eucaryotes; revue dans (334). Cet inhibiteur est efficace contre toutes les caspases et inhibe l'apoptose dans les cellules humaines et de souris. Nous avons observé qu'un pourcentage plus élevé de cellules transfectées par le vecteur p53-spécifiques ont subi l'autophagie par rapport aux cellules transfectées par le vecteur contrôle. La coloration de ces cellules avec un anticorps anti-LC-3 conjugué à le FITC a montré des vacuoles autophagiques dans le cytoplasme. LC-3 est normalement distribuée dans tout le cytoplasme et les cellules montrent une faible coloration lorsqu'elles sont marquées avec un anticorps anti-LC-3 qui diffuse partout dans le cytoplasme. Quand les cellules subissent l'autophagie, LC-3 se clive, se lipide et se concentre dans les couches double-membraneuses des vacuoles autophagiques (335); revue dans (336). Les vacuoles apparaissent sous forme d'agrégats quand elles sont colorées par un anticorps anti-LC-3 conjugué au fluorochrome. En outre, nous avons observé par Western blot la migration rapide de la forme clivée et lipide de LC-3 (désignée comme étant LC-3 II). De plus, l'ajout *in vitro* de 3-MA (3-méthyladénine) à ces cultures cellulaires y réduit considérablement la mort. Il est à noter que 3-MA inhibe les étapes initiales de la formation d'autophagosome en bloquant toutes les classes III de PI-3K (337). Ces résultats suggèrent que le modèle prédominant de mort cellulaire induit par stabilisation et déplétion de p53 dans les cellules humaines est causé par autophagie.

En effectuant des recherches dans la littérature, nous avons constaté qu'un groupe de chercheurs a récemment montré qu'une inhibition de l'expression de p53 par siRNA ainsi que son inactivation par PFT entraine l'autophagie dans une variété de cellules

humaines (338). Toutefois, ces chercheurs n'ont pas déterminé si l'autophagie induite donnait lieu à la mort cellulaire. En fait, l'autophagie est considérée comme un processus de protection cellulaire, puisqu'elle fournit une source d'énergie pour un temps limité dans des conditions de famine.

C'est pour cette raison que l'utilisation du terme «mort cellulaire autophagique" est fortement déconseillée dans la littérature.

Au départ, pifithrine-α (PFT) a été découverte comme étant de petites entités chimiques qui inhibent la mort cellulaire induite par p53 dans les cellules humaines en réponse aux stimuli d'endommageant d'ADN (329, 339). Comme mentionné ci-dessus, PFT inhibe la transcription des gènes cellulaires induite par p53. Utilisée en culture à des concentrations plus élevées (> 30 uM), PFT est toxique *in vitro* pour les cellules en croissance. Il a été rapporté que PFT a une faible solubilité dans des solutions aqueuses et précipite lentement à partir de ces solutions comme un produit de condensation appelé Pifithrine-β. Une forme en anneau se forme pendant le processus de condensation (voir Figure 20). Contrairement à sa forme α, pifithrine-β, n'a aucun effet sur p53, mais elle peut induire la mort cellulaire via la voie d'AhR (*Aryl Hydrocarbon Receptor*) (340). Toutefois, elle est très toxique *in vitro* pour les cellules en croissance.

Depuis sa découverte, l'utilisation de PFT a été favorisée en tant que thérapie d'appoint pour le soulagement des effets secondaires toxiques associés à la chimio-radiothérapie et aux cancers (339). Ce traitement a été choisi car les autres thérapies anticancéreuses mènent inévitablement à la stabilisation et à l'activation de p53. P53 activée initie un nouveau programme de transcription des gènes et induit l'expression de plusieurs gènes impliqués dans l'arrêt du cycle cellulaire lors de la sénescence et l'apoptose, par exemple, p21, BAD, BAX, PUMA, etc ; revue dans (308). L'inhibition de la transcription médiée par p53 prévient de nombreux effets toxiques induits par les thérapies anticancéreuses.

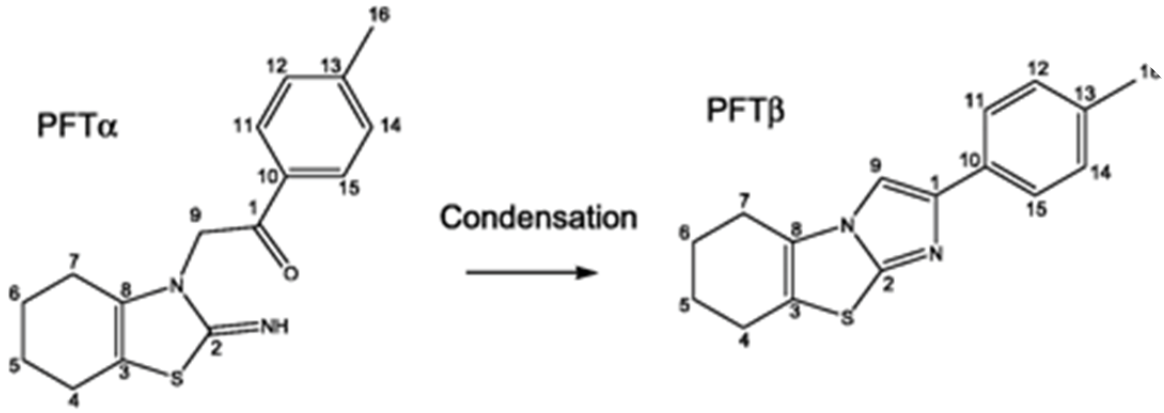


Figure 20 : **Structure de PFT-α et son produit de condensation PFT-β.**

D'après (341)

Depuis sa découverte en 1998, de nombreuses études ont démontré des effets protecteurs de la PFT contre l'apoptose induite en réponse aux dommages d'ADN et suite à l'activation de p53 (339, 342, 343). Cependant, seules quelques études ont déjà examiné les effets de PFT *in vitro* sur les cellules en croissance dans des conditions normales, c'est à dire, sans stress. Nous sommes les premiers à se rendre compte de ses effets induisant la mort des cellules humaines cultivées dans des conditions normales.

Les effets de PFT induisant la mort cellulaire pourraient s'expliquer par le fait que p53 exprimée à un niveau physiologique basal et celles exprimée sous des conditions de stress aboutissent à différentes conséquences vis-à-vis de la survie cellulaire;

revue dans (136). Normalement, p53 est constamment dégradée par MDM-2, qui elle-même est induite par p53 comme un mécanisme de rétroaction négative. La situation est encore plus compliquée puisque p53 intra-nucléaire et celle intra-cytoplasmique détiennent des fonctions bien distinctes concernant leurs effets sur la survie cellulaire. P53 intra-nucléaires, se produit en réponse au stress, elle exerce des effets pro-apoptotiques en induisant l'expression de BAB, BAX, PUMA, etc, et elle favorise l'autophagie en induisant l'expression du gène DRAM (*Damage Response Autophagy Modulator*). DRAM se localise dans les membranes lysosomales et favorise l'autophagie (344, 345). Toutefois, p53 intra-cytoplasmique, prévient l'autophagie. Par conséquent, sa déplétion conduit à l'autophagie, comme il est suggéré par nos résultats. Fait intéressant, de nombreux réactifs qui induisent l'autophagie entrainent la déplétion de p53 cytoplasmique (136, 309). Pour compliquer encore plus le cadre, il a été démontré que p53 cytoplasmique agit comme une protéine BH-3-only (346). Les protéines BH-3 only représentent un sous-groupe de protéines appartenant à la famille Bcl-2 et qui comprend BIM, PUMA, NOXA, Bid, etc. Ces protéines peuvent se transloquer vers les membranes mitochondriales, lier Bcl-2, causer la polymérisation de Bax et Bak, et conduire à la mort cellulaire par apoptose; revue dans (347). Nos résultats montrent que la mort cellulaire médiée par PFT n'est pas induite par apoptose, puisque l'utilisation d'un inhibiteur des caspases à large spectre ne put l'inhiber. De nombreuses études ont démontré que PFT inactive p53. Ils ont montré qu'en présence de PFT, les radiations gamma et les médicaments endommageant l'ADN n'induisent pas l'expression des gènes cibles de p53 comme les MDM-2, p21, PUMA, etc (136). Cependant, une étude a conclu que PFT ainsi que son produit de condensation, PFT-β, tuent les cellules humaines en raison de leurs effets cytotoxiques directs. Les chercheurs n'ont observé aucun effet de PFT-α ni de PFT-β sur la sur-régulation des gènes p21 et MDM-2 médiée par p53 en réponse aux stimuli induisant le dommage d'ADN (341). Jusqu'à présent, aucune étude indépendante dans la littérature n'a vérifié ces résultats.

Contrairement à la PFT, Nutline-3 prévient la dégradation de p53 en interférant avec son interaction avec MDM-2 (331, 348, 349). Comme il est prévu et tout dépend des fonctions de p53 activée, Nutline-3 entraîne soit l'arrêt temporaire du cycle cellulaire soit l'arrêt permanent (sénescence) ou encore l'apoptose en fonction de l'étendue des dommages d'ADN et des conditions cellulaires (l'état de différenciation, le répertoire des modulateurs apoptotiques), etc. Dans de nombreuses situations, où p53 est fonctionnelle mais qu'elle est tenue inactive, Nutline-3 pourrait favoriser les fonctions de p53 et être utilisé dans des situations cliniques. Par exemple, les lignées cellulaires lymphoblastoïdes (LCL) transformées par EBV, en croissance continue et les cellules du sarcome de Kaposi (KS) infectées par HHV-8 ont toutes les deux des p53 de type sauvage, mais qui sont inactivées par les protéines virales. Le traitement de ces cellules par Nutline-3 provoque la stabilisation et l'activation p53. P53 activée

induit l'expression de nombreux gènes pro-apoptotiques ainsi que l'activation de la caspase-2. Par conséquent, Nutline-3 conduit à la mort cellulaire dans les cellules LCL, KS ainsi que dans de nombreux autres types de tumeurs avec le gène p53 sauvage (276, 348, 350, 351). Il est bien connu que p53 activée induit des gènes pro-apoptotiques.
Cependant, le mécanisme d'activation de la caspase-2 médiée par p53 reste encore énigmatique et pourrait impliquer les PIDDosomes (*PIDD–RA IDD–caspase-2 complex*); revue dans (136, 319, 320).
P53 est l'un des gènes suppresseurs de tumeurs les plus étudiées. Il a été surnommé le «gardien» du génome; revue dans (136). La signalisation et les fonctions effectrices induites par p53 font partie intégrante de la réponse aux dommages d'ADN et des points de contrôle du cycle cellulaire. Son inactivation entraîne une instabilité génomique, une aneuploïdie et l'apparition de cancers. Il n'est pas surprenant que plus de 50% des cancers humains montrent des mutations au niveau du gène p53. Toutefois, p53 n'est pas seulement le gardien du génome. P53 affecte également le métabolisme cellulaire et l'état redox (308). Les souris les mouches et les vers qui ont des p53 knock down, jouissent d'une vie plus longue et d'un vieillissement retardé. Par contre l'activation continue de p53 aussi bien chez les humains que chez les souris et les mouches, conduit à la sénescence et à un vieillissement prématuré (352, 353); revue dans (354). D'autre part, quand p53 est surexprimée normalement et qu'elle soit régulée de façon normale, elle peut augmenter la durée de vie (355). Fait intéressant, quand p53 est à son niveau basal physiologique, elle agit comme un antioxydant et aide à maintenir l'état normal d'oxydoréduction dans les cellules (136). Il est probable que la déplétion du niveau basal de p53 dans une cellule induit un stress oxydant. Ce stress oxydatif, ainsi que l'autophagie accrue peuvent entraîner la mort cellulaire. En effet, nos résultats montrent que l'addition de N-acétylcystéine, un antioxydant, ne réduit pas la mort cellulaire médiée par la PFT. Il est intéressant de constater que cette mort se réduit significativement par des inhibiteurs de la nécroptose et d'autophagie.

Il est à noter que p53 subit de nombreux changements post-traductionnels. Ces changements comprennent la phosphorylation, l'acétylation, la méthylation, la sumoylation, les poly-et mono-ubiquitinations. Il y a au moins 14 résidus sérines qui pourraient être phosphorylés par différentes kinases.
Les conséquences de ces modifications post-traductionnelles ne sont pas toutes comprises (356, 357). Une étude récente a montré que l'ampleur de la réponse p53dépend du nombre de résidus phosphorylés (131). Il est tout à fait concevable que l'ampleur et le type des modifications post-traductionnelles de p53 pourraient conduire à sa localisation dans différents compartiments cellulaires (c'est à dire nucléaire, cytoplasmique ou mitochondriale) et pourraient mener à différentes fonctions touchant la survie cellulaire, l'autophagie, le métabolisme apoptotique, l'état redox et le programme transcriptionel.

Il est tout à fait décevant qu'après plusieurs décennies de recherche, nous sommes encore très loin de saisir toute la complexité de cette protéine.
Une dernière remarque à noter concernant le rôle de p53 dans la réplication virale. Comme indiqué plus haut, les virus suscitent inévitablement la réponse aux dommages d'ADN entraînant ainsi la stabilisation et l'activation de p53. Ils codent également pour différentes protéines dans le but d'inactiver p53; revue dans (161, 328, 358). C'est comme si les virus peuvent mieux se répliquer en l'absence de p53. En fait, il a été démontré que pour plusieurs virus, après la délétion de leurs gènes qui codent pour des protéines inactivant p53, ils arrivent à se répliquer et à tuer les cellules ayant p53 mutée. Ces observations ont conduit à la notion de «virus oncolytiques»; revue dans (359). Ces virus oncolytiques, dont certains sont encore en essais cliniques, pourraient être utiles dans la thérapeutique des cancers dus à des mutations de p53. À cet égard, nous avons observé que HHV-6 ne se réplique pas dans les cellules HSB-2 en présence de la PFT-α. Nous avons observé que les cellules infectées par HHV-6, lorsqu'elles sont mises en culture en présence de PFT-α, sont incapables d'exprimer l'antigène viral précoce. Ces expériences ont été réalisées avec une faible concentration de PFT-α qui n'induit pas d'effets cytotoxiques dans les cellules. Ces résultats suggèrent que p53 pourrait être nécessaire à la réplication au moins pour ce virus. D'autres études sont nécessaires pour vérifier ces résultats. Il serait intéressant de voir si cet agent inactivant p53 a un effet sur d'autres herpèsvirus aussi.

5.3. HHV-6 et l'autophagie

Nous avons montré dans cette partie, que HHV-6 retarde la progression du cycle cellulaire des cellules HSB-2 en culture ainsi que des cellules humaines primaires T CD4 *in vitro* et augmente le pourcentage de ces cellules dans la phase G2 du cycle cellulaire. Il est à noter que les cellules ne subissent pas l'autophagie dans cette phase (360-362). Elles doivent produire plus de protéines et d'organelles, et augmenter par la suite la synthèse des protéines ainsi que les processus anaboliques. Par conséquent, les cellules s'agrandissent et doublent de volume.
Leurs contenus cytoplasmiques se divisent équitablement entre les deux cellules filles lors de la cytocinèse. Si les cellules ne doubleraient pas de taille en G2, les divisions cellulaires auraient pour conséquence une diminution progressive de la taille cellulaire.
Ainsi l'autophagie est une réponse avantageuse pour les cellules hôtes. Elle les protège de la mort puisqu'elle permet à ces dernières de se nourrir de leurs propres constituants, ce qui leurs fournit de l'énergie dans les conditions de pénurie (363).

Un faible niveau basal de l'autophagie se produit dans les cellules pour dégrader les protéines qui ont trop vécu et les vieilles organelles inefficaces comme les mitochondries. Ce faible niveau basal d'autophagie fait partie intégrante du système d'entretien des cellules pour ne garder à l'intérieur que celles en bonne santé. Cependant, les cellules utilisent aussi cette réponse pour enfermer et dégrader les agents pathogènes comme les bactéries et les virus. Ce processus est nommé xénophagie (364). Par contre celui utilisé contre les virus est appelé virophagie, il s'agit d'une tentative de la part des cellules pour se débarrasser du virus envahisseur. Il est de plus en plus évident que l'une des raisons pour laquelle de nombreux virus préfèrent la phase G2, est que durant cette phase, les cellules ont une capacité réduite de monter cette réponse anti-virale autophagique.

L'autophagie des virus et des bactéries envahisseurs contribue également à la présentation des antigènes pathogènes par les cellules présentatrices d'antigène sur les CMH de classe I et CMH de classe II (présentation croisée) des molécules sur les cellules T naïves par voie lysosomale. Ce processus aide également à l'affichage des antigènes auto-phagocytés sur les molécules du CMH de classe I chez les cellules non-présentatrices d'antigène (365, 366).

Pour faire face à la réponse autophagique des cellules, les virus ont développé des stratégies pour contrer non seulement cette réponse, mais aussi pour l'exploiter afin de favoriser leur propre réplication; revue dans (367, 368). Par exemple, l'infection par le VHC (virus de l'hépatite C) induit la réponse autophagique et utilise les structures doubles membranaires induites par l'autophagie pour favoriser la traduction du nouvel ARN viral. Toutefois, l'infection ne permet pas à la réponse autophagique d'avoir le dessus: elle empêche la fusion des lysosomes avec les autophagosomes (ou la maturation des autophagosomes).

Récemment, il ya eu une panoplie d'articles de recherche concernant la manipulation et l'exploitation de la réponse autophagique des cellules par différents virus (367, 368). Toutefois, rien ou presque n'est connu concernant la réponse autophagique des cellules hôtes à l'égard de HHV-6. Nous avons abordé cette question et nous montrons ici pour la première fois que l'infection virale induit la réponse autophagique dans les cellules infectées par ce virus. Nous avons tiré cette conclusion en examinant des vacuoles autophagiques par microscopie confocale après avoir réaliser un marquage intracellulaire des cellules avec un anticorps anti-LC-3 conjugués au fluorochrome. Les vacuoles ont augmenté en nombre et en taille à fur et à mesure que l'infection se dirigea vers les effets cytopathiques. Cependant, les vacuoles des cellules infectées par HHV-6, étaient plus petites et moins nombreuses que celles observées dans les cellules traitées à la rapamycine.

Il est à noter que la rapamycine inhibe la cible de la rapamycine chez les mammifères (mTOR; *mammalian Target of Rapamycin*): une sérine/thréonine

kinase qui joue un rôle important dans le métabolisme cellulaire (369). Ainsi, l'infection virale et la rapamycine exercent un effet autophagique additif dans les cellules HSB-2.
D'autre part, l'addition de 3-MA a diminué sans toutefois inhiber complètement la réponse autophagique induite par le virus.
Le réactif 3-MA inhibe une kinase PI-3K-like Vps-34, dont l'activité est nécessaire à la formation de structures double membraneuses en forme des coupes qui entourent et isolent des structures cytoplasmiques ou étrangères destinées à être autophagées (370). Vps-34 phosphoryle Beclin-1 pour le séparer de Bcl-2 (371).
Les effets de ces deux modulateurs autophagiques (rapamycine et 3-MA) sur la réponse autophagique induite par HHV-6 dans les cellules HSB-2 ont été comme prévu. Nous avons constaté que malgré que HHV-6 induise une réponse autophagique, il empêchait la fusion des
vacuoles autophagiques avec les lysosomes. La fusion des vacuoles autophagique avec les lysosomes est nécessaire pour la formation des autophagolysosomes.
Le pH des autophagolysosomes devient acide, et provoque une activation des enzymes lysosomales, qui finissent par dégrader le contenu des autophagolysosomes (370).
Le processus par lequel les vacuoles autophagiques fusionnent avec les lysosomes
entraînant une dégradation de leurs contenus, est appelé maturation autophagosomale. Nos résultats suggèrent qu'en empêchant la fusion entre les autophagosomes et les lysosomes, le virus prévient la maturation des autophagosomes. Nous pensons que la maturation complète des autophagosomes induite par le virus n'est probablement pas souhaitable pour une réplication virale efficace.
En empêchant la maturation des autophagosomes, le virus empêche également l'affichage (qui pourrait se produire par l'autophagie) des peptides dérivés du virus à la surface des cellules infectées. Nous avons essayé de déterminer les effets potentiels des différents modulateurs autophagiques sur la réplication de HHV-6, pour cela, nous avons infecté les cellules HSB-2 par le virus ou par une préparation du mock, nous les avons lavé avec du PBS pour éliminer le virus résiduel et les avons cultivé *in vitro* en présence d'un véhicule, la rapamycine (qui induit l'autophagie en inhibant mTOR (372), 3-MA (qui inhibe la formation de vacuoles autophagiques en inactivant une kinase IP-3-like (373) ou la bafilomycine A (qui empêche l'acidification des lysosomes en inhibant la pompe à protons vacuolaire, (374)). Douze jours après infection, nous avons récolté les cellules et les surnageants de culture et avons mesuré le nombre de copies d'ADN viral à l'aide d'un RT-PCR en temps réel.
Nous avons constaté d'après ces expériences, que la rapamycine réduit la réplication virale. Le nombre de copies d'ADN viral a diminué à la fois à l'intérieur des cellules que dans les surnageants de culture. Il semblerait que l'autophagie induite par la rapamycine renforce la répression de la réplication de HHV-6.
Il est possible que l'infection virale ne soit pas en mesure de modifier l'autophagie (par exemple, inhiber la maturation des autophagosomes) induite par la rapamycine. Les

effets de l'inducteur autophagique sur la réplication virale ne sont pas dus à ses effets sur la survie cellulaire, puisque la rapamycine n'affecte pas significativement la mort cellulaire dans les cellules mock-infectées. Comme la rapamycine touche de nombreux processus biologiques des cellules humaines autres que l'autophagie, la possibilité que cette drogue affecte la réplication virale en affectant un ou plusieurs processus biologiques autres que l'autophagie ne peut être exclue.

Contrairement à la rapamycine, l'ajout de 3-MA (un inhibiteur des processus précoces impliqués dans l'autophagie) n'a eu aucun effet sur la réplication virale. Toutefois, il a augmenté le nombre des copies d'ADN viral dans les cellules et dans les surnageants de culture, respectivement. Il semblerait que ce médicament favorise la libération des virions à partir des cellules infectées. L'effet du traitement sur la réplication virale globale n'est pas surprenant, puisque ce médicament a réduit, mais n'a pas complètement inhiber la réponse autophagique induite par le virus dans les cellules infectées par HHV-6. Nous sommes incapables d'expliquer comment cette drogue pourrait affecter la libération des virions à partir des cellules. Dans ces expériences, l'effet de la bafilomycine A sur la réplication de HHV-6 dans les cellules HSB-2 a été très intrigant. Cet effet est plus ou moins similaire à ce qui a été observé avec le traitement à la rapamycine. Ce traitement a réduit les copies d'ADN viral à la fois à l'intérieur des cellules que dans les surnageants. Il est à noter que la bafilomycine empêche l'acidification et donc la dégradation médiée par les lysosomes. L'inhibition de cette voie de dégradation des protéines pourrait être nécessaire pour l'élimination de plusieurs protéines virales immédiates précoces et précoces qui sont produites de façon transitoire au cours de la réplication de ce virus. Leur accumulation peut être nuisible à la production des protéines virales tardives, à l'assemblage ainsi qu'à la libération des virions.

Il est également possible qu'une ou plusieurs protéines cellulaires qui s'accumulent dans le cytoplasme suite à l'inhibition de la dégradation médiée par le lysome puissent inhiber la réplication virale. De toute évidence d'autres études sont nécessaires pour vérifier ces hypothèses.

Conclusions

Comme tous les autres virus, HHV-6 a recours à plusieurs stratégies déjouant les mécanismes de défenses cellulaires pour détourner la machinerie de la cellule hôte infectée à son profil et promouvoir sa propre réplication. Une propriété remarquable de ce virus, est qu'il induit une immunosuppression et inhibe la prolifération cellulaire dans les lymphocytes humains. En outre, il induit l'activation ainsi qu'une augmentation de la régulation de p53 dans les cellules qui sont infectées par ce virus. Il est connu que p53 inhibe la progression du cycle cellulaire, l'apoptose ou la sénescence dans les cellules. Dans cette étude nous avons essayé de comprendre l'impact de l'infection des cellules T par HHV-6 sur la progression du cycle cellulaire, surtout que les études ultérieures étaient contradictoires.

Nous avons pu trancher entre ces études et démontrer que l'infection des cellules HSB-2 par la souche GS de HHV-6A permet une accumulation apparente des cellules dans les phases Set G2/M. Cependant, les cellules infectées qui sont traitées par la DNAse micrococcale (qui dégrade l'ADN viral) s'arrêtent dans la phase G2/M. Nous avons trouvé que l'expression de cdc2 ainsi que de son partenaire catalytique la cycline B diminue dans les cellules infectées. Toutefois, nous avons observé une augmentation de l'activité kinase de cdc2 dans ces cellules qui pourrait être due à une protéine virale qui agirait comme un partenaire de cdc2 et qui catalyse son activité. Ceci n'est pas étonnant puisque plusieurs virus codent pour différentes protéines qui interagissent avec des facteurs cellulaires dans le but de modifier et réguler la progression du cycle cellulaire. Nous avons trouvé qu'une protéine virale p41 qui est codée par le gène virale U27, se fixe sur cdc2 et l'active. En plus, nos résultats démontrent que la cycline B se localise dans les noyaux des cellules qui sont infectées par ce virus. Cette localisation nucléaire de la cycline B se produit à la transition G2/M. Cette observation suggère que HHV-6 change le microenvironnement cellulaire comme au début de M. Nos résultats de Western blot indiquent qu'il y a une diminution de l'expression de p21 dans les cellules infectées malgré une induction accrue de p53 dans ces mêmes cellules. Cette diminution de p21 est restaurée par l'utilisation d'un inhibiteur protéosomal et elle est en accord avec le fait que les cellules ne s'arrêtent pas en G1. La diminution de p21 dans les cellules infectées par le virus HHV-6 indique que p53 n'est pas active transcriptionellement dans ces cellules. En accord avec ce résultat, nous avons trouvé par micropuce, que l'expression d'un autre gène cible de p53, GADD-45, est aussi diminuée. Nous avons également observé une diminution globale de l'expression de la securin dans les cellules infectées par ce virus. Il est connu que l'expression de cette protéine cellulaire atteint un pic à la phase M pour garder les chromatides attachés les uns aux autres jusqu'à ce qu'au début de l'anaphase. Une diminution globale de l'expression de la securin dans les cellules infectées par ce virus pourrait conduire à des défauts dans le

temps de séparation et dans la ségrégation des chromatides aux cellules filles. Fait intéressant, nous avons trouvé aussi que HHV-6 entraine une augmentation de la polyploïdie dans les cellules infectées par HHV-6. En outre, nous avons observé plusieurs changements dans l'expression et dans la distribution des protéines du cytosquelette dans les cellules infectées par ce virus. Nous concluons que le virus induit plusieurs changements dans la progression du cycle cellulaire dans les cellules infectées et qu'il modifie leur microenvironnement dans le but de promouvoir sa propre réplication.

Les informations sur la régulation des machineries du cycle cellulaire par différents virus permettent de mieux comprendre la pathogenèse virale. Ces connaissances pourraient aussi nous aider dans le développement de nouveaux médicaments pour contrôler la réplication virale.

Dans une tentative de développer un meilleur système qui soit plus efficace pour la réplication de HHV-6 *in vitro*, nous avons utilisé un siRNA pour inhiber l'expression du gène p53 dans les cellules HSB-2. Étonnamment, nous avons constaté que la transfection du vecteur p53-spécifique, mais pas celle du vecteur contrôle, a causé la mort cellulaire des cellules HSB-2. Des résultats similaires ont été obtenus lorsque p53 a été inactivée dans ces cellules par un inhibiteur pharmacologique, la PFT. Cette mort a été également observée aussi bien dans une lignée cellulaire dotée d'une p53 mutante transcriptionellement que dans des cellules T humaines primaires. Rajoutant plus de complexité à la question, nous avons également observé la mort cellulaire lorsque les cellules ont été traitées avec nutlin-3, un agent pharmacologique qui inhibe la MDM-2 et stabilise et active p53. Ces résultats montrent qu'aussi bien la déplétion que la stabilisation de p53 ont eu un effet négatif sur la survie cellulaire. Nous avons également trouvé que la mort cellulaire pourrait être évitée par des inhibiteurs de l'autophagie et de nécroptose. Fait intéressant, ni l'inhibiteur de caspase, ni le N-acétyle cystéine n'ont pas empêché cette mort. Nos études concernant le rôle de p53 dans les cellules nous ont permis de conclure qu'un niveau basal de p53 est nécessaire pour la survie cellulaire. Ces conclusions expliquent l'augmentation d'attention accordée aux fonctions indépendantes de la transcription et celles cytoplasmiques de p53 dans la survie des cellules.

L'autophagie est un processus important dans la réponse immunitaire. Ce processus permet aux cellules non seulement de dégrader certains de leurs composants mais aussi de détruire les agents pathogènes comme les virus et les bactéries. C'est pour cela que les virus développent des stratégies pour contrer l'autophagie. Comme l'autophagie est régulée dans les cellules dépendamment des phases du cycle cellulaire et que HHV-6 retarde les cellules dans la phase G2/M, nous avons essayé d'investiguer l'autophagie dans les cellules infectées par ce virus. Nous avons montré ici et ce pour la première fois que HHV-6 induit l'autophagie dans les cellules infectées. Nous avons trouvé que le nombre et la taille des vacuoles autophagiques augmentent dans les cellules infectées. Le virus induit le clivage et

la lipidation de LC-3 dans les cellules infectées. Nous avons montré que ce virus inhibe la fusion des vacuoles autophagiques avec les lysosomes et la maturation des autophagosomes. Comme prévu, le traitement des cellules infectées par HHV-6 par le 3-MA inhibe l'expansion de l'autophagie, par contre leurs traitement par la rapamycine l'augmente. D'après nos résultats de RT-PCR en temps réel, l'induction de l'autophagie par la rapamycin diminue le nombre de copies d'ADN dans les cellules infectées par rapport aux cellules infectées et non traitées. Alors que l'ajout du 3-MA augmente le nombre de copie d'ADN viral dans le surnageant. Notre étude ouvre de nouvelles perspectives sur le déroulement de l'autophagie dans les cellules humaines infectées par HHV-6.

6 Références bibliographiques

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Annexe

Role of HSV-1-induced apoptosis and autophagy on viral replication and cell death in human monocytic cells

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Abstract

HSV-1 is a ubiquitously occurring pathogen that infects humans early in childhood. The virus persists as a latent infection in dorsal root ganglia and frequently become reactivated in humans under conditions of stress. Monocytic cells constitute an important component of the innate and adaptive immune responses. We show here that the virus induces expression of FasL and apoptosis in human monocytes, macrophages and an established cell line THP-1. The infection increased expression of FasL at the transcriptional level. The addition of a broad spectrum caspase inhibitor reduced cell death but increased viral replication in the virus-infected cell cultures. The virus also induced autophagy in the infected cells late in the replicative cycle. The addition of an authophagy inhibitor at the time of infection or 5 hours after the infection inhibited cell death and viral replication. Similar results were used when an autophagy inducer was added to the cell cultures at the time of infection. However its addition 5 hours after the infection increased viral replication. These data suggest that inhibitors of autophagy may be considered as potential antiviral agents. Our study provides novel insights concerning the impact of apoptosis and autophagy modulators on the viral replication in human monocytic cells.

Key words: Apoptosis, Autophagy, FasL, HSV-1, Monocytes, Macrophages, THP-1, U937.

Introduction

Herpes Simplex virus type 1 (HSV-1) is a ubiquitously occurring human herpes virus that infects humans early in life (reviewed in (10, 11, 53)). It is a member of the α-Herpesvirinae subfamily. Primary infections with the virus usually occur in early childhood and are mild or symptom-less. However, the infected humans can never rid off the virus and become life-long carriers. The virus travels from the skin nerve endings to dorsal root ganglia, where it becomes latent. The latent infections frequently become reactivated under conditions of stress, immunosuppression, physical trauma or exposure to UV radiation (40). These reactivations are often manifested as painful blisters or "cold sores" at mucocutaneous junctions of lips. The morbid condition is called Herpes labialis. The virus may also infect cornea and cause keratitis. These morbid conditions cause considerable discomfort and represent a serious health problem. Primary and reactivated latent infections may rarely cause encephalitis especially in neonates and immunocompromised persons (53). Effective anti-HSV drugs have been developed, however the emergence of drug-resistant viruses has also been documented especially in immunocompromised individuals (reviewed in (30)). Unfortunately, effective vaccines are not available against the virus. Monocytes and macrophages represent important cellular elements of the immune system. In response to a viral infection, they release a variety of pro-inflammatory cytokines and chemokines, and recruit inflammatory cells to the site of infection. Activated macrophages phagocytose pathogens and immune complexes, and present viral antigens to other immune cells. Unlike epithelial cells, in which HSV-1 prevents apoptosis and causes cell death with predominant features of necrosis, HSV-1 infects monocytic cells with different degrees of permissiveness and seems to induce their cell death via apoptosis (8, 9, 31). However, little is known about the mechanism of this virus-induced apoptosis, its consequences for antiviral immunity as well as for viral replication.

Autophagy (macroautophagy) is a host response to a viral infection that has attracted much attention in recent years. Autophagy is a physiological ubiquitin-like process in which long-lived proteins and

old cellular organelles are sequestered into double membrane-bound vacuoles (autophagosomes), which fuse with lysosomes (autolysosomes; reviewed in (16)). The contents of the autophagosomes are degraded and recycled. It appears that via autophagy, host cells tend to degrade viruses and other pathogens. However, viruses have evolved strategies to counter this response and may use it to their own advantage (reviewed in (36, 46)). The occurrence of autophagy and degradation of the virus has been reported in HSV-1-infected human cells (12, 51). The virus encodes two proteins ICP34.5 and US11, which can counter the starvation and PKR (double stranded RNA-induced protein kinase)-mediated autophagy (39, 50). Furthermore, ICP34.5 has been demonstrated to bind beclin-1, a cellular protein known to be essential for the induction of autophagy in human cells (4). The induction of autophagy-inhibiting viral proteins suggests that the virus interferes with and modifies the autophagic response of the host cells. How the autophagic response affects viral replication and cell death remains unknown. We addressed these questions and show here that HSV-1 infection induces apoptosis in human monocytic cells via inducing expression of FasL on their surface. Interference with this pathway prevents cell death but enhances viral replication in these cells. We also show that the virus induces autophagy in late stages of the infection. The induction of autophagy in HSV-1 infected macrophages (with rapamycin) at the time of infection reduces viral replication but its induction 5 to 6 hours' post-infection increases viral replication. The inhibition of autophagy in the infected cells at both time points post-infection reduced cell death and viral infection. These observations provide valuable insights on the relevance of apoptosis and autophagy to viral replication and death of the virus-infected cells.

Materials and Methods

Cell cutlure

THP-1, U937 and Vero cells were obtained from ATCC. All cell lines were cultured in the culture medium RPMI-1640 containing 10% FCS, 2 mM L-glutamate, 100 units per ml of penicillin and 100 µg per ml streptomycin (Gibco, Burlington, ON) at 37°C in 5% CO2 humidified atmosphere. Human peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of blood over Ficoll-Hypaque (Pharmacia, Montreal, QC) and washed with the culture medium without FCS and antibiotics. Human monocytes were isolated from PBMC by negative selection using a commercially available kit (StemSep, Stem Cell Technology, Vancouver, BC). Purity of the isolated cells was verified by fluorescence-activated cell sorting (FACS) analysis using FITC-conjugated anti-CD14 antibodies (BD Biosciences, Mississauga, ON), and was always ≥ 95%. The monocytes were also isolated by adherence to plastic surface. After removing the non-adherent cells, the adherent cells were induced to differentiate into macrophages by culturing them into the culture medium containing 10% FCS, 5% human AB serum and 2 ng/ml of recombinant human (rh) granulocyte-macrophage colony stimulating factor (GM-CSF, BioSource, Camarillo, CA) for 5 days.

Antibodies and reagents

The antibodies used in this study were purchased: mouse anti-human FasL from BD Biosciences (Mississauga, ON), PE-conjugated mouse anti-FasL from eBioscience (San Diego, USA), mouse anti-Fas agonist from Upstate (Temacula, CA), rabbit anti-human Bcl-XL from Cell Signalling Technology (Boston, USA), mouse anti-human Bcl-2 from Calbiochem (San Diego, USA), and mouse anti-human glyceraldehyde phosphate dehydrogenase (GAPDH) from Ambion (Austin, TX). Recombinant human TNF-α was purchased from eBioscience. Acyclovir (9-(2-hydroxyethoxymethyl) guanine) was obtained from Calbiochem (San Diego, CA). 3Methyladenine (3-MA) and Rapamycin were obtained from Sigma-Aldrich (Saint-Louis, MI) and Calbiochem,

respectively. Cell permeable broad spectrum caspase inhibitor N-benzyloxycabonyl-Val-Ala-As-fluoromethylketone (Z-VAD-fmk) was obtained from Calbiochem (San Diego, CA) and diamidino-phenylindole (DAPI) was obtained from Sigma-Aldrich (Saint-Louis, MO).

Virus preparation

Cell-free HSV-1 (McIntyre strain) was prepared from cell lysates and culture supernatants of HSV-1-infected Vero cells as described earlier (2, 19, 20). The virus-containing culture medium was passed through 0.45 um filters (Corning, Lowell, MA) and then centrifuged at 14000 x g for 90 minutes at 4˚C. The viral pellets were resuspended in the RPMI-1640 medium, titrated and stored in aliquots at -80˚C until used. The mock viral preparations were made like the viral preparations but from the culture supernatants obtained from uninfected Vero cells growing in culture. To prepare non-infectious HSV-1, the virus preparation was irradiated by a 60-minutes exposure to a UV source that delivered UV at 400 μJ per second. The loss of infectivity of the irradiated virus preparation was also tested by its plaque-forming ability on Vero cell monolayers. The viral stocks were aliquoted and kept at -80°C. In some experiments, we used a recombinant fluorescent HSV-1 (KGFP-gB), which produces enhanced green fluorescent protein (EGFP) tagged with the ectodomain of its gB as described (41). The fluorescent virus replicates less efficiently and produces smaller plaques as compared to its wild type parent strain KOS. The virus was also produced on Vero monolayers as described earlier (2, 41).

Titration of HSV-1

The viral preparations were titrated by plaque-forming assay as described elsewhere (2, 33). Briefly, 2 million Vero cells were incubated in 100-mm-diameter culture dishes and infected with 100 µl of logarithmically diluted virus preparations for 2h at 37°C with intermittent shaking. After removing the viral inoculum, 5 ml of 1% methylcellulose was added and the cells were incubated at 37°C in humidified 5% CO2 atmosphere. After 3 days, methylcellulose was removed by gentle suction, and the cell monolayers were washed with phosphate-buffered saline (PBS; pH 7.2) and fixed with formalin (diluted 1:5 with PBS). The fixed cells were stained with 0.1% crystal violet, and the plaques were counted under an inverted microscope. The plaques were counted only for the dilutions giving 20-100 plaques per culture dish. The average number of plaques per culture dish was multiplied by 5 and the dilution factor to determine the number of PFU per milliliter. The viral stock used contained 10^8 PFU per ml. The supernatants from various infected cells were titrated with the TCID50 method. Briefly, $2x10^4$ cells were dispensed in 150 ul of the culture medium in duplicate in the wells of a flat bottomed 96 wells plate. Then the diluted (10to 10) supernatants were added in 50 µl volume to each well. The microcultures were incubated for 3 days at 37 in 5% CO2. The microcultures were examined under the microscope and TCID50 was calculated by the Spearman-Karber formula (17).

HSV-1 infection

For HSV-1 infection, cell pellets were incubated with the virus preparation with different M.O.I at 37°C for 90 min in 15 ml tubes, washed thrice, and resuspended in the culture medium. The cells were incubated at 37°C in a humidified 5% CO2 atmosphere for different period of times.

Western-blotting

The expression of different proteins within cells was analyzed by Western blotting as described in our earlier publications (3). Briefly, 5×10^6 cells were incubated in the culture medium with and without treatment as detailed in individual experiments. Cells were then washed with phosphate-buffered saline and lyzed in a lysis buffer containing Tris HCl (pH 6.8; 50 mM), sodium dodecyl sulfate (SDS) (2%), leupeptin (1 mg/ml), phenylmethylsulfonyl fluoride (1 mM), and pepstatin (1 mg/ml). The cell lysates were clarified by centrifugation at 14.000 × g for 15 min. Protein concentrations were determined in the lysates by using a commercial kit (Pierce, Nepean, ON)**.** Forty micrograms of the lysate proteins were mixed with 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer containing 1 mM dithiothreitol, boiled, run on 12% SDS-PAGE gels, and electroblotted onto polyvinylidene difluoride membranes (Immobilon P; Millipore) by semi-dry electroblotting system (Dryblot, Bio-Rad). After blockage of the membranes in 1% casein in PBS for 2h at room temperature, they were incubated on a shaker with protein-specific antibodies, i.e., anti-FasL or anti-GAPDH, at 4°C over-night. The protein bands were revealed by autoradiography by using biotinylated goat anti-mouse antibodies and a commercial chemiluminiscent kit (Vectastain ABC-AmP; Vector, Burlington, ON). Individual bands on the X-ray films were quantified into arbitrary units by densitometry.

Flow Cytometry

For immunostaining for FasL, 10^6 cells were pelleted, washed with PBS and resuspended in residual liquid. PE-conjugated anti-human FasL antibodies were added onto the cell pellets as recommended by the manufacturer. The cells were incubated on ice for 45 minutes on ice with intermittent shakings. After the incubation, stained cells were washed thrice with PBS 0.05%

BSA and 0.002% sodium azide, and resuspended in PBS containing 2% paraformaldehyde. The cells were analyzed by flow cytometry using FACS Calibur (BD Bioscience, San Diego, USA).

Detection of Apoptosis

Apoptotic cells were detected by their ability to stain with FITC-conjugated Annexin-V and PI using a commercial kit (Cell Death Kit; BD Biosciences). Briefly, 10^5 cells were washed in PBS and resuspended in 100 µl of 1X Annexin-V binding buffer. Cells were then stained with 10 µl of FITC-conjugated Annexin-V and 10 µl of PI for 15 minutes at room temperature in the dark. Cells were then diluted with 400 µl of 1X binding buffer as recommended by the manufacturer and analysed by flow cytometry using FACS Calibur (BD Biosciences). In all cases, the stained cells were analysed within 15 minutes after the staining.

Transfection and FasL reporter gene assay

The ability of HSV-1 to activate human FasL gene was investigated by the virus-induced transcriptional activation of a human FasL promoter-reporter gene construct. The construct was made using the plasmid pGL-3 basic (Promega) in which a 511 bp region upstream the FasL gene start codon was fused with the firefly luciferase gene as described (34). The cells were transiently transfected with 5 µg of the construct DNA using DMRIE-C transfection reagent (Invitrogen, Burlington, ON). After the transfection, the cells were divided into two halves: one half was infected with HSV-1 and second half was used for mock infection. The cells were harvested 18 hours post-infection and subjected to the luminescence analyses by using a commercial kit (Luciferase Reporter Assay Kit, Promega, Nepean, ON).

Detection of autophagy

To detect autophagy, cells were transfected transiently with an expression plasmid that encoded LC3-RFP; a fusion protein between monomeric Red Fluorescent Protein (RFP) and LC-3 (the microtubule-associated protein-1 Light Chain-3). The plasmid was obtained from W. Beron (Universidad Nacional de Cuyo-CONICET, Argentina) and has been described earlier (1, 23). During autophagy, LC-3 becomes conjugated with phosphatidylethanolamine and is localized in phagophores and autophagosomes (26). In normal cells, the protein appears diffusely and uniformly distributed throughout the cytoplasm whereas it appears in punctuate form in autophagic cells. The distribution pattern of the LC3-RFP was examined by confocal microscopy.

Confocal microscopy

Vero cells were cultured on coverslips that were placed in 6-well culture plates and were pretreated with poly-L-lysine (Invitrogen, Burlington, ON) for 10 minutes. After infection with KGFP-gB, cells were fixed with 4% (wt/vol) paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.05% Triton X100, and stained with 4'-6-Diamidino-2phenylindole (DAPI). The cells were washed three times with PBS. THP-1, U937, and primary cells were separately infected with KGFP-gB virus, cultured in the medium in suspension and stained with DAPI as for Vero cells. The DAPI-stained cells were mounted onto glass slides with Vectashields (Vector, Burlington, ON) and examined with a confocal microscope (LSM 510 META, Zeiss, Oakville, ON).

Statistical Analysis

Standard Student “t” test and one way ANOVA were performed using the software PRISM (GrapPad, San Diego, CA, USA). The differences yielding p values ≤ 0.1 were deemed statistically significant.

Results

HSV-1 infection induces the cell death of THP-1 but not of U937 cells

In order to learn about the interactions of HSV-1 with human monocytic cells, we infected THP-1 and U937 *in vitro* with the virus. As shown in Figure 1A, HSV-1 infection resulted in the death of THP-1 cells as compared to the mock-infected cells (80% vs. 18%). Interestingly, the HSV-1 infection of U937 cells did not affect their viability significantly. In order to confirm that this effect on the cells was due to HSV-1 infection and not due to the presence of any other soluble factor in the viral preparation, we infected THP-1 cells with UV-irradiated HSV-1 or filtered viral preparation. As shown in Figure 1B, the infection of THP-1 cells with the UV-irradiated virus abrogated the virus-induced cell death resulting in a percentage of the dead cells equivalent to that seen in the mock-infected cells. Furthermore, we passed the viral preparation through a 0.1 μm filter, which should have retained the viral particles present in the preparation. As shown in Figure 1B, the 0.1 μm filter-passed preparation failed to cause death in THP-1 cells as compared to the 0.45 μm filter-passed one where the virus was not retained. These results show that HSV-1 infection affects the two human monocytic cell lines differently; it infects and kills THP-1 but not U937 cells. We investigated if the cell death observed in THP-1 cells was dose and time-dependent. We infected THP-1 cells with HSV-1 *in vitro* at different M.O.I (ranging from 0.001 to 100) for different lengths of time. Our data show that the percentage of dead cells correlates with the quantity of virus used for the infection (Figure 1C). The numbers of dead cells were not significantly different in U937 cells between infected and mock-infected cells at all M.O.I. In order to know if the virus-induced cell death was time dependent, we infected THP-1 cells with a low M.O.I (0.1) and counted dead cells at different time points (6 to 72 hours). The cell death

started 12 hours post-infection and continued increasing until 72 hours post-infection. It is noteworthy that by 24 hours post-infection, during which HSV-1 completes its first round of infection, most of the cell death has already occurred. On the contrary, cell death in U937 cells remained stable during the entire culture period (Figure 1D).

The HSV-induced cell death in THP-1 cells occurs via apoptosis

We performed experiments in order to determine whether the cell death in the THP-1 cells was due to apoptosis or necrosis. For this purpose, we stained the cells with DAPI and examined them under a fluorescent microscope. As shown in Figure 2A, the nuclei of HSV-infected THP-1 cells showed chromatin condensation characteristic of apoptosis. We also stained cells with PI and FITC-conjugated Annexin V to discriminate the cell death between apoptosis and necrosis. As shown in the Figure 2B, THP-1 cells infected with the virus, but not U937 cells became positive for the two markers. It is noteworthy that the cells undergoing apoptosis and necrosis differ from each other temporally with respect to the sequence of expression of these two markers. The cells undergoing necrosis become positive for Annexin V and PI simultaneously. However, the cells undergoing apoptosis show a time lag between staining for these two markers. First, they show staining for Annexin V and become PI-positive only several hours later (28). In this experiment, we treated THP-1 cells with anti-Fas agonistic antibody and used them as positive control for apoptosis. We also treated these cells with TNF-α and used them as positive control for necrosis as described (28). As shown in Figure 2C, HSV-1 infected THP-1 cells followed the pattern of sequential staining with FITC-Annexin V and PI, as seen in the case of the cells undergoing apoptosis due to treatment with anti-Fas antibodies. They shifted progressively from the lower left quadrant (PI-negative/FITC-Annexin V-negative cells) to the lower right quadrant (PI

negative/FITC-Annexin V-positive cells, t = 9 hours) and with time to the upper right quadrant (PI-positive/FITC-Annexin V-positive cells, t = 18 hours). These data show that HSV-1 induces the apoptosis of THP-1 cells.

HSV-induced apoptosis is inhibited by a broad spectrum caspase inhibitor

Caspases are the main initiator and effector molecules that cause apoptosis in human cells. Therefore, we investigated whether a broad-spectrum cell-permeable caspase inhibitor (Z-VADfmk) can block HSV-induced cell death in THP-1 cells. As shown in Figure 2D, addition of the inhibitor reduced the virus-induced cell death significantly ($p < 0.01$) at all time points.

Inhibition of apoptosis in HSV-infected THP-1 cells enhances viral replication

Since HSV-induced apoptosis could be inhibited by caspase inhibitors, we sought to determine the effect of the caspase inhibitor on the viral replication. As shown in Figure 2E, addition of a broad-spectrum caspase inhibitor enhanced HSV production in THP-1 cells at all time points examined. These results show that preventing apoptosis in THP-1 cells enhances the viral replication.

HSV-1 replicates in THP-1 cells with lower efficiency as compare to Vero cells

In order to determine if HSV-1 infection was productive and resulted in the release of infectious virions, we infected THP-1, U937 and Vero cells with a recombinant HSV-1, KGFP-gB, as described in the Materials & Methods' section and analysed them for the expression of the virus-encoded gB-GFP by confocal microscopy. Vero cells were used as a positive control for the viral replication due to their high permissivity for productive HSV-1 infection. When the infected cells were examined three hours post-infection, no fluorescence signal was observed in all types of cells suggesting that any residual virus from the inoculum could not have given false positive results (data

not shown). When examined 18 hours post-infection, green fluorescence was observed in Vero and THP-1 cells but not in U937 cells (Figure 3A). The numbers of GFP-positive cells were significantly lower ($p < 0.01$) in THP-1 cultures as compared with the Vero cultures (5.0 ± 4.6 vs. 86.0 ± 10.6 percent positive cells, respectively). Furthermore, the intensity of the signal emitted by the gB-GFP was much lower in the virus-infected THP-1 cells as compared to the infected Vero cells (Figure 3A). No fluorescence was observed in U937 cells. These data suggest that HSV-1 replicates less efficiently in THP-1 cells as compared with Vero cells. We also verified these results by measuring viral titers in the cell culture supernatants obtained from HSV-1-infected Vero, THP-1 and U937 cells. For this purpose, the cells were infected with HSV-1 for 24 hours and the culture supernatants were titrated by plaque-forming assay as described in Materials and Methods. The cells were washed four times after the infection and the 4th wash was also titrated for determining residual HSV-1 particles remained in the cell cultures. As shown in Figure 3B, THP-1 cells produced three times less viral particles as compared to Vero cells ($0.9 \pm 0.1 \times 10^6$ PFU per ml vs. $2.6 \pm 0.1 \times 10^6$ PFU per ml, respectively). These data show that THP-1 cells produce infectious virions but were less permissive to the viral replication as compared with Vero cells. We also collected cell-free culture supernatants from the three infected cells and determined their ability for causing cell death in THP-1 cells. As shown in Figure 3C, the culture supernatant-caused cell death correlated with their viral titers.

Viral replication is needed for HSV-induced apoptosis of THP-1 cells

In order to know if the viral replication was needed to induce the apoptosis in THP-1 cells, we infected cells with and without adding acyclovir for 24 hours. Acyclovir, a guanine analogue, is one of the most commonly used drugs against HSV-1 infections (38). It inhibits replication of HSV-1 by inhibiting the activity of the viral DNA polymerase. As shown in Figure 3D, no fluorescence was seen in THP-1 and Vero cells infected with the KGFP-gB virus when they were treated with 300 μg/ml of acyclovir as compared to untreated cells. These data confirm the efficiency of the treatment with acyclovir in abrogating the viral replication in THP-1 and Vero cells. When we treated HSV-1-infected THP-1 cells with the drug, it inhibited the virus-induced cell death (1% vs. 41% dead cells; Figure 3E). These data clearly show that the viral replication is needed to induce apoptosis in THP-1 cells.

The virus-induced apoptosis in THP-1 cells is mediated by the Fas/FasL interactions

In order to understand the molecular mechanism involved in the HSV-1-induced cell death in THP-1, we attempted to block the apoptosis by using anti-FasL antibodies that inhibit Fas/FasL interactions. The addition of anti-FasL antibody significantly reduced apoptosis in HSV-1infected cells as compared to isotype-matched control antibodies (Figure 4A). Only 29% of infected cells incubated with anti-FasL antibody were positively stained with FITC-Annexin-V compared to 68% for cells treated with the isotype control antibody. The experiment was repeated and dead cells were counted using the trypan blue exclusion assay. As shown in Figure 4B, the viability of THP-1 cells infected with HSV-1 and treated with 1 μg/ml anti-FasL antibody was increased by almost 30% as compared to isotype-treated cells (43 ± 0.17% vs. 73 ± 1%, respectively) ($p < 0.01$). We conclude from these experiments that the Fas/FasL pathway represents one of the mechanisms involved in the viral-induced THP-1 apoptosis.

HSV-1 infection induces *de novo* expression of FasL expression on THP-1 cells

Based on these observations, we investigated whether HSV-1 infection induced FasL expression in THP-1 cells. For this purpose, we infected THP-1 and U937 cells for 18 hours and analysed FasL expression by flow cytometry. As shown in Figure 5A, the infection induced FasL expression on the surface of THP-1 cells but not in U937 cells. Interestingly, no FasL expression was observed after treating HSV-1-infected-THP-1 cells with acyclovir (Figure 5B), suggesting that the viral replication is required to induce the expression of FasL. These results also confirm our above-mentioned observation that the viral replication was needed to induce the THP-1 cell apoptosis. We then investigated whether the viral infection was inducing expression of the FasL gene. For this purpose, we determined the effect of the viral infection on the transcription of a reporter gene placed under the control of FasL promoter as described in the Materials & Methods section. As shown in Figure 5C, the FasL promoter activity is significantly increased in HSV-1 infected cells as compared to mock-treated cells (46057 ± 686 vs. 6870 ± 70 RLU, respectively; $p < 0.01$). Similarly to our flow cytometry data, no FasL promoter activity was observed after treating HSV-1-infected-THP-1 cells with acyclovir. We also compared the levels of FasL expression in HSV-1 and mock-infected THP-1 cells by Western blots eighteen hours after the infection. As shown in Figure 5D, HSV-1 infection caused increased expression of FasL in the virus-infected cells. The results also suggest that both THP-1 and U937 constitutively expressed FasL intracellulary. Since FasL is known to be shed into culture medium by proteolytic cleavage of the surface-expressed FasL (52), we measured soluble (s) FasL by using a commercial ELISA kit.

Surprisingly, no significant increase in the concentrations of the sFasL could be detected in the supernatants harvested from THP-1 and U937 cells that were mock-or HSV-1-infected (data not shown). Consequently, our data suggest that the viral infection leads to FasL expression on the surface of THP-1 cells which is implicated apoptosis of these cells. In separate experiments, we compared the expression of Bcl-2 and Bcl-XL between HSV-infected and mock infected THP-1 cells 18 hours postinfection by Western blots and found very little differences between them (data not shown).

HSV-1 infects and induces apoptosis in purified human monocytes via the Fas/FasL pathway

We further wanted to know whether HSV-1 infects and causes apoptosis in purified human monocytes. For this purpose, we isolated monocytes from PBMC as described in the Materials & Methods' section and infected them with KGFP-gB and examined them under a fluorescent microscope. The virus underwent replicative cycle evidenced by the expression of the protein GFP-gB (data not shown). These results conclusively show permissivity of human monocytes to HSV-1. As shown in the Figure 6A, the viral infection caused apoptosis in these cells and the neutralizing anti-FasL antibody significantly reduced this apoptosis in HSV-1-infected cells as compared to control antibodies-treated cells. Indeed, only 23% of infected cells incubated with anti-FasL antibody were positively stained with Annexin-V compared to 92% for infected cells alone and 90% for cells treated with the isotype control antibody. The experiments were repeated and dead cells were counted using the trypan blue. As shown in Figure 6B, the viability of monocytes infected with HSV-1 and treated with 1 μg/ml of anti-FasL antibody was significantly increased (21 ± 0.55% vs. 86 ± 3%, respectively) ($p < 0.001$). Furthermore, we also determined

the expression of FasL on the surface of HSV-1 and mock-infected human monocytes. As shown in Figure 6C, HSV-infected cells expressed FasL on their surface. Taken together, these data show that HSV-1 also induces apoptosis in human monocytes and Fas/FasL pathway represents the main mechanisms of this apoptosis. We also determined the effect of the caspase inhibitor on the viral replication in human monocytes. The caspase inhibitor significantly ($p < 0.01$) reduced virus production in these cells when the virus titre was measured in culture supernatants at 24, 48 and 72 hours after infection (Figure 6D).

HSV-1 infection induces apoptosis in monocyte-derived macrophages

In vivo monocytes differentiate into macrophages, which play an important role in regulating inflammation and immune responses in body tissues in response to pathogens. In order to investigate whether HSV-1 infects and induces apoptosis in these cells, we generated macrophages from purified human monocytes as described in the Materials & Methods. We infected the macrophages with HSV-1 or UV-irradiated HSV-1 for 24 hours and observed by standard microscopy if the virus was able to induce cytopathic effects in them. Interestingly, cytopathics effects (rounding, detachment and cell death) were observed in the cells after 24 hours' infection with the virus but not with the mock or UV-inactivated viral preparations (Figure 7A). In fact many of the infected cells became detached and were floating in the culture media leaving visible empty surfaces in cell monolayers whereas the monolayers were intact in mockinactivated HSV-1-infected cells. These data show that HSV-1 is able to induce cell death in human macrophages. In contrast to monocytes, the addition of anti-FasL antibodies did not reduce cell death in HSV-infected monocyte-derived macrophages (data not shown). We further wanted to know whether monocyte-derived macrophages were permissive to HSV-1. For this purpose, we infected them with KGFP-gB and examined them under a fluorescent microscope.

As for isolated monocytes, the virus underwent replicative cycle evidenced by the expression of the protein GFP-gB (data not shown). We stained the macrophages 12 and 24 hours post-infection and determined the expression of FasL on their surface by flow cytometry. The infection induced expression of FasL on the surface of human macrophages (Figure 7B). Western blots for FasL expression showed that mock-infected macrophages also expressed this molecule and HSV-infection increased this expression (Figure 7C). Finally we determined whether HSVinfected and FasL-expressing human macrophages could induce death of Fas-positive T cells in co-cultures. As shown in Figure 7D, HSV-infected macrophages induced death of positive human T cells and the death could be inhibited significantly by anti-FasL antibodies ($p < 0.01$).

HSV-1 induces autophagy in human monocytic cells

It is well known that host cells respond to viral infection by inducing autophagy, and viruses tend to inhibit this response. In this connection, HSV-1 is known to encode an early protein ICP-43.5 that inhibits autophagy by binding with beclin-1 (37). Despite this knowledge, the effect of autophagy modulators on the viral replication has not been reported. To answer this question, we first investigated the status of autophagy in HSV-1 infected THP-1 cells. For this purpose, we used THP-1 cells that were transiently transfected with an expression plasmid that contained RFP-LC3 gene under the HCMV early promoter. Sixteen hours after the transfection, the cells were infected with the KGFP-gB, washed and examined under confocal microscope. It is noteworthy that RFP-LC3 appears as diffuse red staining in normal cells but gives punctuate staining in autophagic cells. The puncta indicate autophagic vacuoles in whose walls LC3 accumulates. The Figure 8A shows typical LC3-transfected and HSV-infected autophagic cells at different time points after the infection. No autophagic cells were observed until 5 hours after the infection. The autophagic cells appeared late in the course of the infection. These data suggest that HSV-1 infection is not able to fully inhibit autophagic response in human cells. In separate experiments, we determined the effects

of Rapamycin (which inhibits mTOR and induces autophagy) and 3-MA (which inhibits a type III PI-3K kinase VPS34 and inhibits autophagy) on the viral replication. As shown in Figure 8B, the rapamycin-induced autophagic vacuoles were trefoilic and much larger compared to the ones induced by the viral infection. Furthermore, 3-MA decreased and rapamycin increased the size of the virus-induced vacuoles. When these reagents were added to HSV-1-infected cells at the time of infection (T=0h), each of them inhibited HSV-1-induced cell death. The effect of 3-MA was more pronounced as compared to that of Rapamycin (Figure 9A). When these cells were examined for FasL expression, 3-MA decreased but Rapamycin increased the virus-induced expression of FasL on the cell surface. Both reagents had no effect on the expression of FasL in mock-infected cells (Figure 9B). These data suggest that Rapamycin and 3-MA per se do not affect FasL expression in these cells. They most likely induce their effects via modulating HSV-1 replication in these cells. Therefore, we sought to determine the effect of these reagents on the viral replication. When these reagents were added to HSV-1-infected cell cultures at the time of infection (T=0h), each of them reduced viral replication in THP-1 cells. Rapamycin inhibited the viral replication more than 3-MA as judged by the green fluorescence emitted by the GFP-gB (Figure 9C). When the reagents were added to the cell cultures 5 hours after the infection, 3-MA reduced but Rapamycin increased the virus production as determined by the viral titers in the culture supernatants (Figure 9D). These data suggest that 3-MA inhibits HSV-1 replication if added to the cell culture irrespective of the time of infection whereas Rapamycin reduces HSV-1 replication if added at early time points and increases it if added to the cell cultures at 5 hours or later time points after the infection.

Discussion

We have shown here that HSV-1 productively infects human monocytes, macrophages as well as a monocytic human cell line THP-1. These conclusions were reached both using a wild type HSV-1 as well as a recombinant virus in which the coding sequences for Green Fluorescent Protein (GFP) were fused in frame with those of gB of the virus (41). It is noteworthy that gB is transcribed as a late (γ1) gene. GFP expression could be clearly seen under a confocal microscope in human monocytes, macrophages and THP-1 cells. Furthermore, these cell types also produced infectious virions as shown by their ability to infect Vero cells; which are known to be very permissive for replication of HSV-1. Previous studies showed that monocytes were resistant to HSV-1 infection and only become susceptible to it upon differentiation towards macrophages (6, 7, 48). Another study showed that human peritoneal macrophages become partially permissive to the viral infection upon treatment with thioglycollate and fully permissive upon prior infection with Corynebacterium parvum (47). We show here that monocytes and monocyte-derived macrophages as well as a monocytic cell line THP-1 are susceptible to infection with the virus, albeit with different degrees of permissiveness. The virus replication is much lower in these cells as compared with Vero cells. Furthermore, we show here that another human monocytic cell line U937 is not permissive to HSV-1 replication. These results are in agreement with those of Feng et al. (14), who showed that the virus did not replicate in these cells but was able to induce activation of NF-κB in them. The cell line, however, also becomes permissive to the viral replication upon differentiation with phorbol myristate acetate, but not with dimethyl sulfoxide or all-trans retinoic acid (48). The cell line expresses the Herpesvirus entry mediator (HVEM), a specific receptor for gD of the virion. The viral glycoprotein gD binds HVEM and activates NF-κb (45). Collectively, these data suggest that human monocytes, macrophages and certain moncytic cell lines can be productively infected with HSV-1. We show here that monocytic cells undergo apoptosis upon infection with HSV-1, and this occurs due to the virus-induced expression of FasL on the surface of these cells. The addition of anti-FasL antibodies

reduces the HSV-1-induced cell death in our study. Similar effects of the virus were reported earlier on human monocyte-derived macrophages (5, 22). We also show here that FasL-expressing, HSV-1-infected human monocytic cells can kill Fas-positive human T cells. Given a wide spread expression of Fas on the surface of human cells, the HSV-1-induced expression of FasL on human monocytic cells could have two consequences: Firstly, it may induce death of the virus-infected cells via apoptosis, and secondly, the infected cells may kill Fas-positive immune cells (e.g., T cells, neutrophils, Natural Killer cells) and evade antiviral immune response. The first consequence may benefit the host by causing early death of the infected cells and hence may result in reduced viral production. Indeed our results show that treating HSV-1-infected monocytic cells with a wide spectrum caspase inhibitor reduces cell death, and increases viral titers in the culture medium. The second consequence (countering antiviral immune response) would certainly benefit the virus in the *in vivo* setting. In this respect, we have shown that the virus-induced apoptosis is a host beneficial response as its inhibition with a broad spectrum caspase inhibitor or with anti-FasL antibodies decreases cell death but increases viral replication. This conclusion is supported by an *in vivo* study with HSV-2, the virus which is very closely related to HSV-1, showing that the virus causes more deaths and replicates to higher titers in mice lacking Fas or FasL gene as compared to the wild type mice (24). The FasLpositive $CD4^{+}$ T cells were shown to play a protective role in this study.

We show here for the first time that HSV-1 enhances FasL expression in monocytic cells at the transcriptional level, as the viral infection stimulates FasL promoter and increases expression of the human FasL promoter-driven reporter gene. The virus has been shown to induce FasL expression and fratricide death in activated human $CD8^+$ T lymphocytes (43). The virus induced FasL expression on various cell types in the eye when mice were infected via anterior chamber of the eye. The infection resulted in enhanced apoptosis of various cell types in the eye and brain of the infected mice (42). A relatively recent study has shown that HSV-1 induces expression of FasL on neonatal, but not on adult, neutrophils (13). The study noted hastened death of the virusinfected neonatal neutrophils that could be inhibited with antagonistic anti-Fas or anti-FasL antibodies. Interestingly, HSV-1 does not induce FasL expression in all cell types. For example, the infection induced cell death in immature dendritic cells not by inducing expression of FasL, but rather by causing enhanced proteasomal degradation of long form of the cellular FILCE (or pro-caspase 8) inhibitory protein (c-FLIP-L) (27, 35). In our studies, replication of HSV-1 is absolutely essential for the HSV-1-mediated induction of FasL expression and cell death in human monocytic cells. Treating the infected cells with acyclovir, which inhibits the viral DNA polymerase and viral DNA replication (38), prevents cell death. This suggests the viral DNA and/or late viral proteins may be responsible in inducing FasL expression and cell death in these cell types. Several early and immediate early proteins of HSV-1 have been shown to induce apoptosis in different human cell types, although their exact mode of action remains unknown. The proapoptotic action of these early viral proteins is countered by subsequently expressed gene products, e.g., α-2, US3, gJ, etc. The net result is that HSV-1 infected cells become resistant to several exogenous death-inducing stimuli (osmotic shock, thermal shock, Fas, TNF-α and C2 ceramide) (15, 18, 44). Furthermore a micro RNA encoded by exon 1 of the LAT gene of HSV-1 protects neuroblastoma cells from apoptosis by down-regulating TGF-β and SMAD3 expression (21). Interestingly the resistance of HSV-1-infected cells to the death-inducing stimuli depends upon the cell type. Furthermore, the pro-apoptotic and anti-apoptotic

effects of different viral proteins also varied with the cell type. It appears that these viral proteins exert their effects by interacting with different cellular factors. The differential expression of these factors in different human cells may be responsible for differential effects of the viral proteins with respect to their effect on cell death. We have shown here that HSV-1 replicates in human monocyte-derived macrophages (MDM) and induces FasL expression at their surface. The virus-infected cells become rounded and detach from monolayers and die. The cell death in these cells could not be prevented by the addition of anti-FasL antibodies, despite the fact that these cells express FasL. The lack of Fas-FasL mediated cell death in these cells may be due the fact that these cells grow in monolayers and do not interact with each other via Fas-FasL. The inability of anti-FasL antibodies to reduce their death suggests that their ultimate death may be due to virus-induced changes intrinsically as it happens with epithelial cells (15). We have demonstrated in this study that the virus-infected FasL-expressing macrophages are able to induce apoptosis in Fas-positive cells that interact with them. Thus the expression of FasL on these cells may enable them to evade killing by invading Natural Killer (NK) cells and HSV-1-specific CTL that may invade the virus-infected tissues. Thus the virus-induced expression of FasL may be more important for the infected cells in evading natural and virus-specific adaptive immunity of the host. With respect to autophagy, we have shown here that human monocytic cells respond to HSV-1 infection via autophagy. Our results show that treating cells with 3-MA (a chemical inhibitor of VPS34 and autophagy) inhibits HSV-1-induced cell death in human monocytic cells (49).

By infecting RFP-LC3-transfected cells with GFP-gB-expressing recombinant HSV-1 in the presence and absence of 3-MA and Rapamycin (which inhibits mTOR and induces autophagy) (32) and examining them under a confocal microscope, we show that autophagic cells start appearing in the cell cultures as early as 4-5 hours post infection. Their number increases as the infection progresses until they start dying 12-20 hours post-infection. It is relevant to mention here that HSV-1 is known to carry at least two genes its genome, US11 and ICP34.5 whose products can suppress autophagy. Of these genes, US11 encodes a serine/threonine kinase that counters PKR-induced autophagy (39). The ICP34.5 encoded protein has a similar function. In addition, it also targets beclin-1 and prevents autophagy. ICP34.5 mutant HSV-1 infected cells exhibit more numerous autophagosomes (4). Since autophagy protects neurons, its inhibition via ICP34.5 is a major factor in the virus-mediated neuropathology in HSV-1-infected mice. Consequently, ICP34.5 mutant viruses are attenuated in their neurovirulence (37). In our hands, 3-MA and Rapamycin inhibit and enhance the virus-induced autophagy in these cells, respectively. The virus-induced autophagy appeared to be morphologically different from the one induced by Rapamycin. The autophagic vacuoles (aggregates of RFP-LC3) were relatively big and appeared more widespread in the cell cytoplasm. In the virus-induced autophagic cells, the vacuoles were mostly located in the perinuclear region and contained DNA. In some of the cells, whole cellular DNA seemed to have disappeared. In agreement with our observations, a recent study has shown that HSV-1-induced autophagic vacuoles arise from the nuclear membranes (12). We show here how modulation of the autophagic response in monocytic cells affects HSV-1 replication and cell death in human monocytic cells. Both Rapamycin and 3-MA, when added separately to HSV-1-infected cell cultures, reduce cell death. Unlike the caspase inhibitor, both of these reagents decreased HSV-1 replication when they were added to the virus-infected cell cultures at the time of infection. This decreased viral replication in the cells may explain reduced cell death in the presence of these autophagy modulators. The autophagy inhibitor (3-MA) decreased the viral replication even when added 5 hours post-infection. However, unlike

3-MA, addition of Rapamycin to the virus-infected cell cultures 5 hours post-infection increased the viral titers in the culture medium. We explain these results by assuming that the virus inhibits autophagy in the early part (first 4-5 hours) of its replicative cycle but uses this process to promote its replication the later part of the cycle. That explains why the addition of rapamycin at the time of infection reduces viral replication but increases it when added 4-5 hours post-infection. The addition of the autophagy inhibitor at both early and late time points prevents autophagy and reduces the viral titers in the culture medium. Taken together these results suggest that autophagy prevents viral replication at early time points but promotes it at later time points. Thus, autophagy inhibitors may be useful as suppressors of HSV-1 replication. Earlier studies showed inhibitory or no effects of autophagy on HSV-1 replication in mouse embryonic fibroblasts (25, 37, 51). The inhibition of autophagy would also inhibit presentation of viral antigens to immune cells and dampen antiviral immune responses. Thus *in vivo* autophagy may promote early immune-mediated clearance of the virus as documented by a recent study (29). This study used mutant and WT viruses in mice but did not observe the direct effect of autophagy modulators. We show here that rapamycin enhances and 3-MA reduces FasL expression on HSV-1-infected but not on mock-infected human monocytic cells. This suggests that the effects of rapamycin on FasL expression are mediated via its effects on the virus. Some viral gene product may be needed to link FasL expression with FasL expression. Our study provides novel insights about HSV-1-induced apoptosis and autophagy in human monocytic cells. Our results suggest that autophagy inhibitors could be of potential use in inhibiting viral replication and enhancing antiviral immunity.

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Figure legends

Figure 1. Infection with HSV-1 induces THP-1 cell death in a dose and time-dependent manner. (A) THP-1 and U937 cells were infected with HSV-1 (M.O.I = 5) or Mock-infected for 2 hours at 37°C. Cells were then washed and put in culture for 24 hours. After 24 hours, total cells were counted and dead cells were discriminated using trypan blue coloration. (B) Cells were infected as described above with HSV-1, with the HSV-1 preparation previously passed through 0.45 µM and 0.1 µM filters or with UV-treated viruses. (C) THP-1 and U937 cells were infected with different concentrations of HSV-1 (M.O.I) for 24 hours. For each condition, total cells were counted and dead cells were discriminated using trypan blue coloration. (D) THP-1 and U937 cells were Mock or HSV-1-infected with a low M.O.I (0.5) and cells were incubated at 37°C for different period of time. For each time points, total cells were counted and the percentage of dead cells was calculated using trypan blue coloration. Three stars on the figure represent $p < 0.001$.

Figure 2. HSV-1 infection induces THP-1 cells apoptosis. (A) THP-1 and U937 cells were infected and put in culture for 24 hours. After 24 hours, cells were permeabilized and DNA was stained with DAPI. The fluorescence was observed by confocal microscopy. (B) THP-1 and U937 cells were Mock and HSV-1-infected (M.O.I = 5) for 2 hours at 37°C and put in culture for 18 hours at 37°C. After 18 hours, cells were harvested and stained with Annevin-V FITC and Propidium Iodide. (C) THP-1 cells were treated with 100 ng/ml TNF-α (to induce their necrosis) and 1 µg/ml of a monoclonal anti-Fas agonist antibody (to induce their apoptosis) or infected with HSV-1 for 3, 9 and 18 hours. At each time points, the cells were harvested and stained with Annevin V-FITC and Propidium Iodide. (D) THP-1 cells were infected with HSV-1 (M.O.I = 1) or Mock-infected for 2 hours at 37°C. Cells were then washed and put in culture for different length of time with or without 10 µg/ml of a broad spectrum caspase inhibitor (Z-VAD). For each time points, total cells were counted and dead cells were discriminated using trypan blue

coloration. (E) For each time points, cell culture supernatants from these infections were also harvested and titered for their contents in newly produced HSV-1 particles using a standard TCID50 titration method as described in Materials and Methods.

Figure 3. HSV-1 replication occuring in THP-1 cells is needed for inducing their apoptosis.

(A) THP-1, U937 and Vero cells were infected with a fluorescent HSV-1 (KGFP-gB) for 2 hours at 37°C. Cells were then extensively washed and put in culture for 18 hours. After this time, cells were harvested, permeabilized and DNA was stained with DAPI. The fluorescence was observed by confocal microscopy. (B) THP-1, U937 and Vero cells were infected with HSV-1 for 24 hours. After 24 hours, supernatant of these cell cultures were harvested and titrated using the standard Plaque-Forming Unit method as described in Materials and Methods. The 4^{th} wash after infection was kept at -20°C and was used as control for the innoculum background. (C) THP-1, U937 and Vero cells were infected with HSV-1 for 24 hours. After 24 hours, supernatant of these cell cultures were harvested, filtered through a 0.45 µM filter and treated or not with UV before infection of fresh THP-1 cells for 24 new hours. After this second infection, total cells were harvested and dead cells were counted using trypan blue. The 4^{th} wash after infection was kept at -20°C and was also used as control for the innoculum background. (D) THP-1 cells were infected as described above with a fluorescent HSV-1 (KGFP-gB). Cells were then extensively washed and put in culture for 18 hours with or without 100 µM of Acyclovir. After this time, cells were permeabilized and DNA was stained with DAPI. The fluorescence was observed by confocal microscopy. In the same experiment, cells were also harvested and stained with Annevin V-FITC and Propidium Iodide.

Figure 4. The Fas/FasL apoptotic pathway is involved in the HSV-1-induced THP-1 apoptosis. (A) THP-1 cells were mock and HSV-1-infected as described above for 18 hours at 37°C with and without 1 µg/ml of a neutralizing monoclonal antibody anti-FasL (Anti-FasL). Mouse IgG (1 µg/ml) (Control Ab) were also used as control. After 18 hours, cells were harvested and stained with Annevin V-FITC and Propidium Iodide. (B) This graph correspond to the percentage of dead THP-1 cells counted using the trypan blue coloration of dead cells after 18 hours of incubation with the virus. Two and three stars on the figure represents $p < 0.01$ and $p < 0.001$, respectively.

Figure 5. HSV-1 infection of THP-1 cells induces FasL expression. (A) THP-1 and U937 cells were infected with HSV-1 for 18 hours as described earlier. After 18 hours, cells were harvested and Fc-Receptors were blocked with 1 µg of mouse IgG. The cells were then stained using a monoclonal antibody anti-FasL PE-conjugated and analysed by flow cytometry. (B) THP-1 and U937 cells were infected as described above and treated with or without 100 µM of Acyclovir. After 18 hours, cells were harvested and Fc-Receptors were blocked with 1 µg of mouse IgG before staining with a monoclonal antibody anti-FasL PE-conjugated. Cells were then analysed by flow cytometry. (C) THP-1 cells were transfected by a construct corresponding to the FasL promoter region (-511 before ATG) followed by a luciferase reporter gene. SV-40-luc (Positive control) and Basic-luc (Negative control) constructs were also tranfected. Twelve hours after transfection, cells were infected with HSV-1 and Mock for 18 hours with or without 100 µM of Acyclovir. After 18 hours, cells were washed and lysed using a passive lysis buffer and the

luminescence was measured as described in Materials and Methods. (D) Cell lysates from THP-1 and U937 cells that were Mock (lane 1 and 3) and HSV-1-infected (lane 2 and 4) for 18 hours were analyzed by Western blotting using a monoclonal anti-FasL antibody. Individual bands were quantified by densitometry and the ratios between the bands densities of the FasL proteins and GAPDH are also indicated. Three stars on the figure represents $p < 0.001$.

Figure 6. HSV-1 infection of human isolated monocytes induces their apoptosis via the Fas/FasL pathway. (A) Isolated human monocytes were infected with Mock and HSV-1 for 18 hours with and without addition of isotype control and anti-FasL antagonistic antibodies (1 μg/ml each). After 18 hours, cells were stained with FITC-conjugated Annexin V and Propidium Iodide, and analyzed by flow cytometry. The cell death was also measured in counting dead cells using the trypan blue exclusion assay (B). (C) Monocytes were infected with HSV-1 for 18 hours as described earlier and treated with or without 100 μM of acyclovir. After 18 hours, cells were harvested and Fc-receptors were blocked with 1 μg of mouse IgG. The cells were then stained using a monoclonal antibody anti-FasL PE-conjugated and analysed by flow cytometry. (D) Monocytes were infected with HSV-1 at low M.O.I (1) or Mock-infected for 2 hours at 37°C. Cells were then washed and put in culture for different length of time with or without 10 μg/ml of a broad spectrum caspase inhibitor (Z-VAD). For each time points, total cells were counted and dead cells were discriminated using trypan blue coloration.

Figure 7. HSV-1 infection of human macrophages induces FasL expression and the apoptosis of co-cultured Fas positive cells. (A) Monocytes were isolated from PBMC by adhesion and differentiated in macrophages for 5 days in RPMI 10% FCS + 5% Human AB serum and 2 ng/ml GM-CSF. After 5 days, monocyte-derived macrophages were Mock or HSV-1 infected for two hours at 37°C. Infection with UV-treated HSV-1 was performed as control. After 2 hours of infection, cells were washed and put in culture for 24 hours. The cytopathic effect was observed by standard microscopy after 24 hours of culture. (B) Macrophages were infected with HSV-1 for 12 and 24 hours as described earlier. Cells were then harvested and Fc-receptors were blocked with 1 μg of mouse IgG. The cells were then stained using a monoclonal antibody anti-FasL PE-conjugated and analysed by flow cytometry. (C) Cell lysates from macrophages that were Mock (lane 1) and HSV-1-infected (lane 2) for 24 hours were analyzed by Western blotting using a monoclonal

anti-FasL antibody. Individual bands were quantified by densitometry and the ratios between the bands densities of the FasL proteins and GAPDH are also indicated. (D) Macrophages were infected with mock or HSV-1 for 15 hours as described earlier and fixed with PBS 2% paraformaldehyde for 30 minutes. CEM cultures with or without 1 μg/ml anti-FasL or control antibodies (mouse IgG) were then added for 24 hours. After 24 hours, floating CEM cells were harvested, stained with Propidium Iodide (PI) and analyzed by flow cytometry. Percentages of apoptotic CEM cells determined by PI positivity are shown.

Figure 8. HSV-1 infection induces autophagy in THP-1 cells. (A) THP-1 cells were transfected with the LC3-RFP plasmid construct for 16 hours and were then infected with a fluorescent HSV-1 (KGFP-gB) for 2 hours at 37°C. Cells were washed and put in culture. After different time points after the infection, cells were permeabilized, stained with DAPI and observed by a confocal microscope. Note the co-localization of HSV-1 GFP-gB and RFP-LC3 in the infected THP-1 cells 8 hours post-infection. (B) The effects of 3-MA and rapamycin on autophagic vauoles induced by the virus 8 hours post-infection. The reagents were added to the cell cultures immediately after the infection. The cells undergoing autophagy are indicated by white arrows.

Figure 9. The viral-induced autophagy increases THP-1 cells death and FasL expression in the late stages of the infection. (A) THP-1 cells were infected with HSV-1 (M.O.I = 5) or Mock-infected for 2 hours at 37°C. Cells were then washed and put in culture for 24 hours with or without 10 µg/ml 3-Methyl Adenine (3-MA) or 10 µg/ml Rapamycin. After 24 hours, total cells were counted and dead cells were discriminated using trypan blue coloration. (B) THP-1 cells were infected and treated as described above. After 18 hours, cells were harvested and Fc-Receptors were blocked with 1 µg of mouse IgG before staining with a monoclonal antibody anti-FasL PE-conjugated. Cells were then analysed by flow cytometry. (C) THP-1 cells were were infected with a fluorescent HSV-1 (KGFP-gB) for 2 hours at 37°C. Cells were then extensively washed and treated as described in (A) for 18 hours. After this time the fluorescence for the gb-GFP was observed by confocal microscopy. (D) THP-1 cells were infected with HSV1 (M.O.I = 5) for 2 hours at 37°C. Cells were then washed and put in culture for 12 hours with or without 10 µg/ml of 3-Methyl Adenine (3-MA) or 10 µg/ml of Rapamycin at T=0 hours post-infection (T=0h p.i) or T=5 hours post-infection (T=5h p.i). After 12 hours, cell culture supernatants from these infections were harvested and titered for their contents in newly produced HSV-1 particles using a standard TCID50 titration method as described in Materials and Methods. One and two stars on the figure represents $p < 0.1$ and $p < 0.01$, respectively.

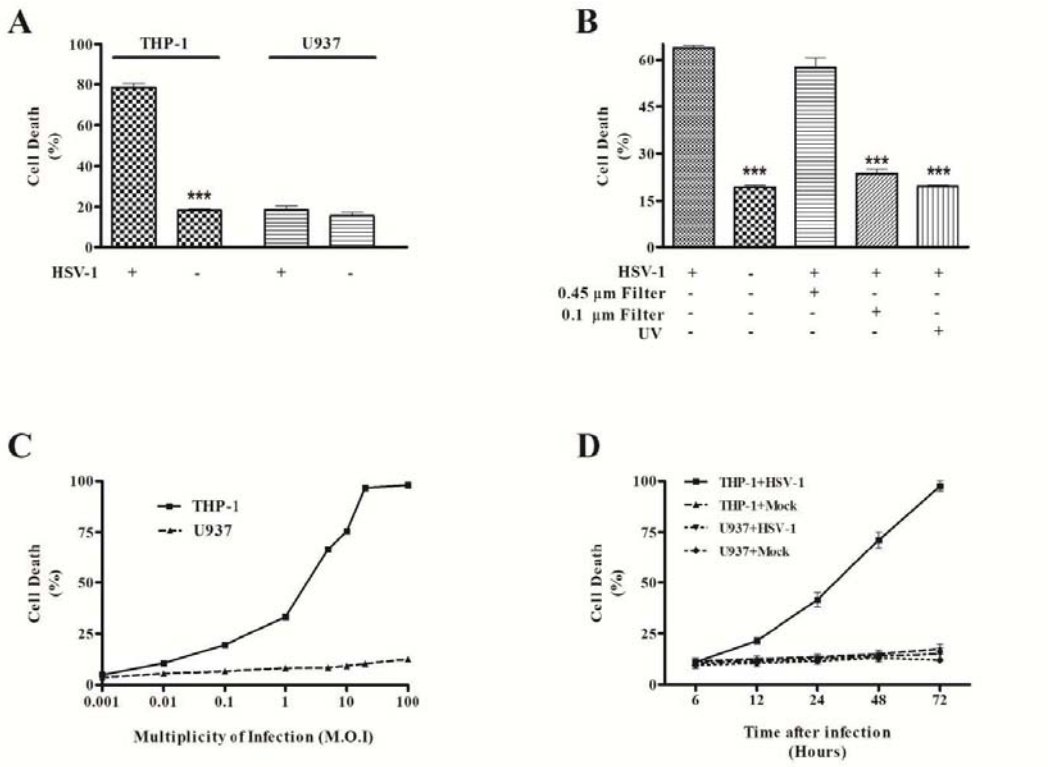

A
THP-1
U937
Cell Death (%)
100
80
60
40
20
0
***
HSV-1 + - + -
B
Cell Death (%)
60
45
30
15
0
***
***
***
HSV-1 + - + + +
0.45 µm Filter - - + - -
0.1 µm Filter - - - + -
UV - - - - +
C
Cell Death (%)
100
75
50
25
0
THP-1
U937
0.001
0.01
0.1
1
10
100
Multiplicity of Infection (M.O.I)
D
Cell Death (%)
100
75
50
25
0
THP-1+HSV-1
THP-1+Mock
U937+HSV-1
U937+Mock
6
12
24
48
72
Time after infection (Hours)

Figure 1

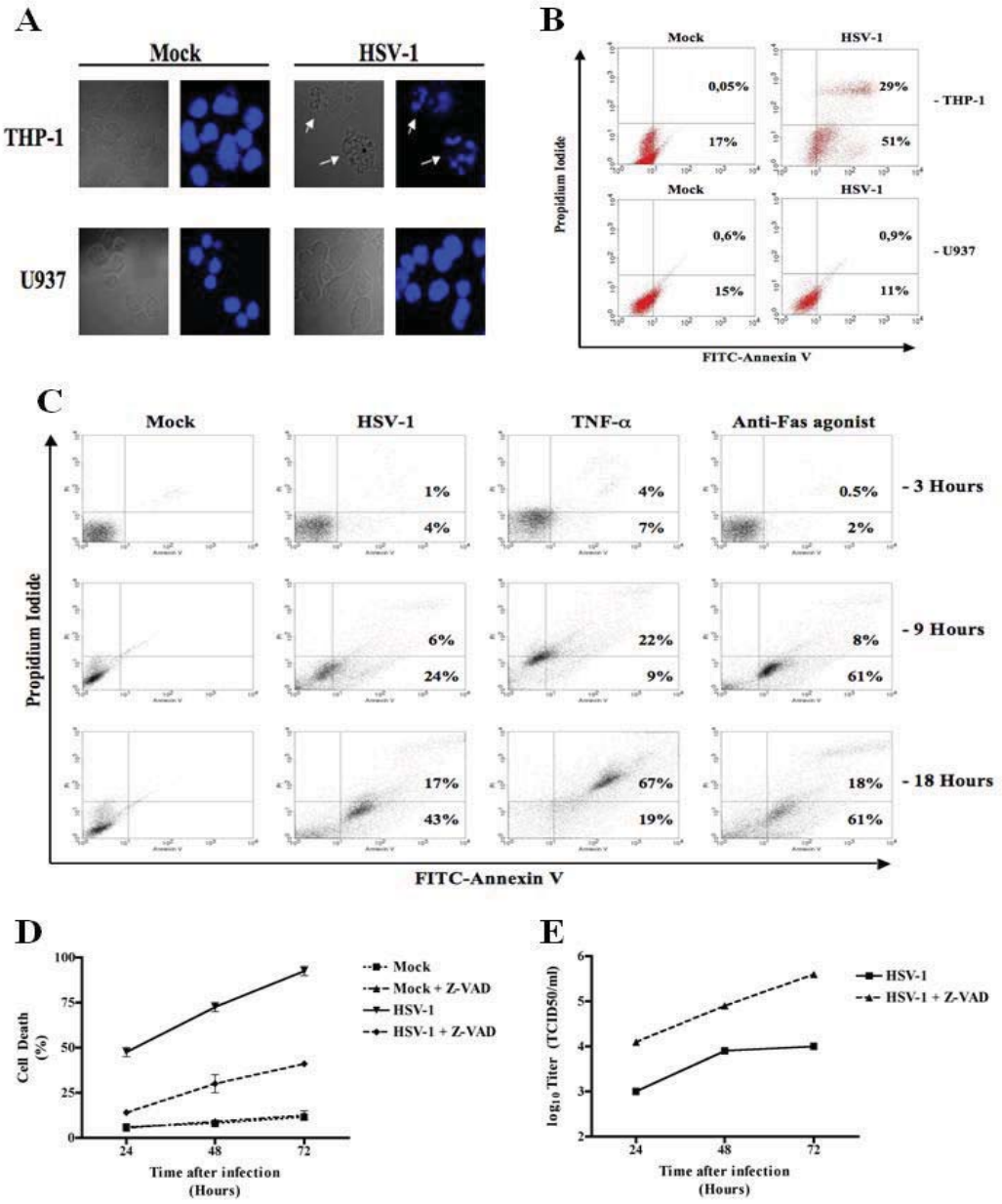

A
Mock
HSV-1
THP-1
U937
B
Mock
HSV-1
0,05%
29%
17%
51%
- THP-1
Mock
HSV-1
0,6%
0,9%
15%
11%
- U937
Propidium Iodide
FITC-Annexin V
C
Mock
HSV-1
TNF-α
Anti-Fas agonist
1%
4%
0.5%
4%
7%
2%
- 3 Hours
6%
24%
22%
9%
8%
61%
- 9 Hours
17%
43%
67%
19%
18%
61%
- 18 Hours
Propidium Iodide
FITC-Annexin V
D
Cell Death (%)
Mock
Mock + Z-VAD
HSV-1
HSV-1 + Z-VAD
Time after infection (Hours)
E
log10 Titer (TCID50/ml)
HSV-1
HSV-1 + Z-VAD
Time after infection (Hours)

Figure 2

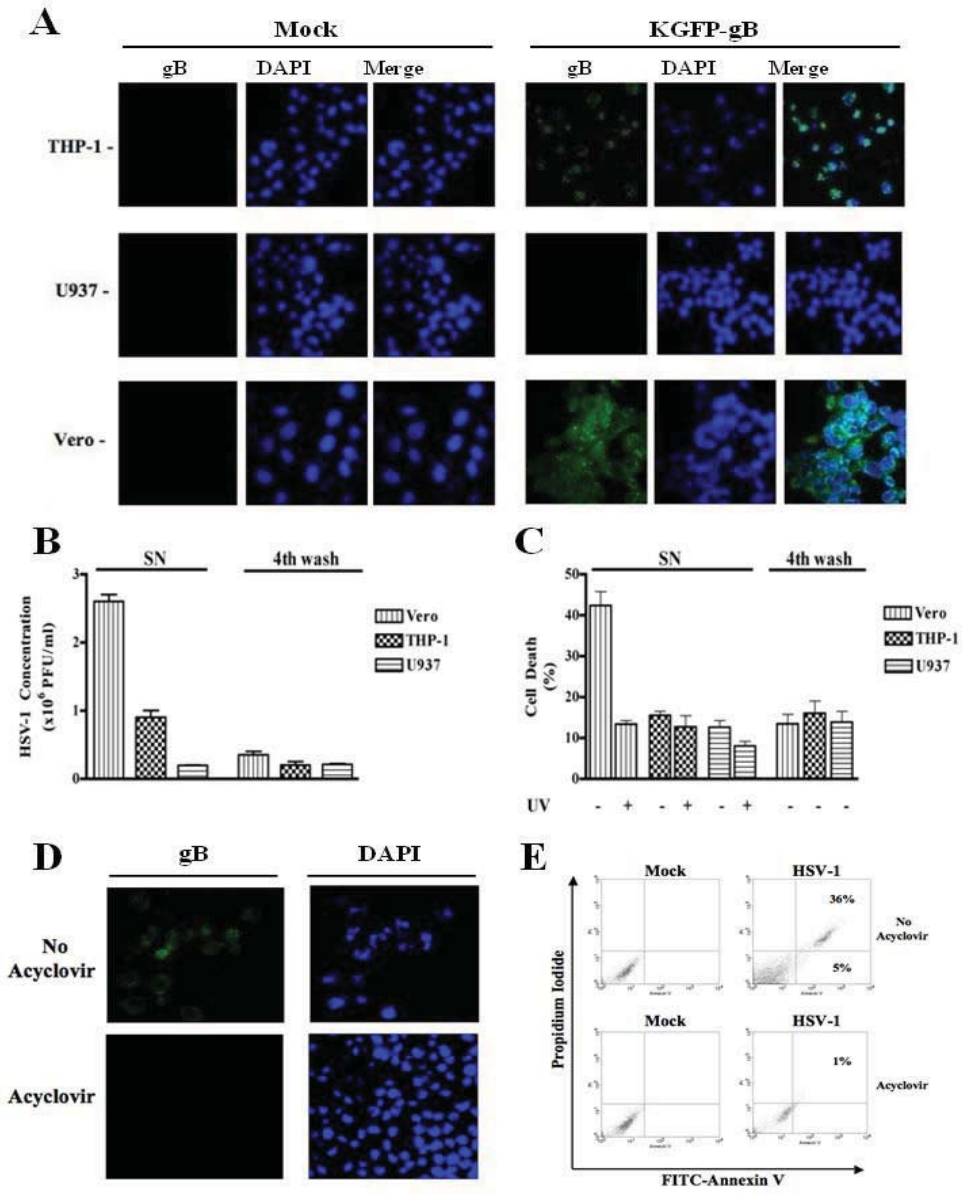
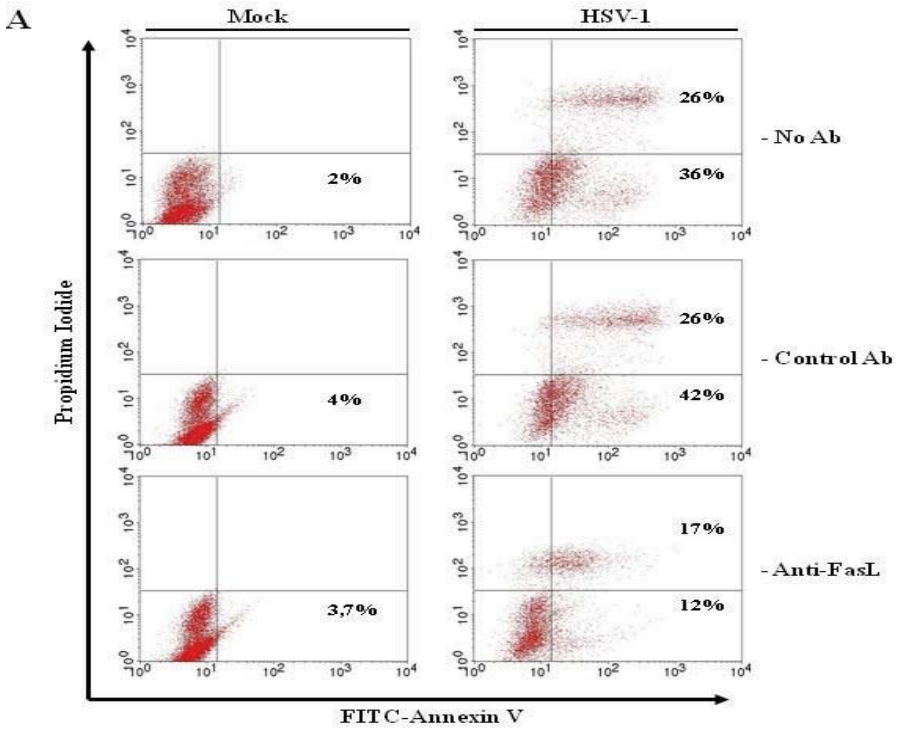

A
Mock
KGFP-gB
gB
DAPI
Merge
THP-1 -
U937 -
Vero -
B
SN
4th wash
HSV-1 Concentration (x10^6 PFU/ml)
Vero
THP-1
U937
C
SN
4th wash
Cell Death (%)
UV
D
gB
DAPI
No Acyclovir
Acyclovir
E
Mock
HSV-1
36%
5%
1%
No Acyclovir
Acyclovir
Propidium Iodide
FITC-Annexin V

Figure 3


A
Mock
HSV-1
- No Ab
- Control Ab
- Anti-FasL
2%
26%
36%
4%
26%
42%
3,7%
17%
12%
Propidium Iodide
FITC-Annexin V

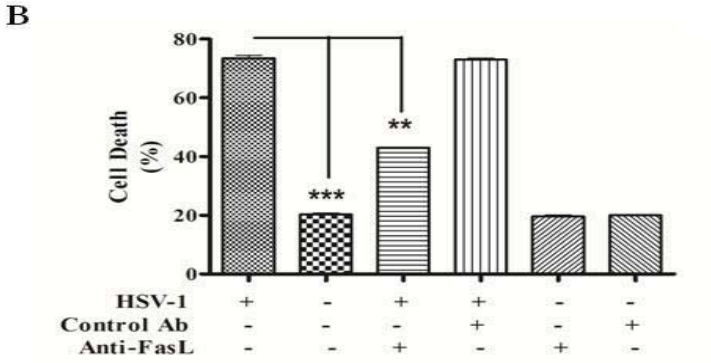

B
Cell Death (%)
80
60
40
20
0
***
**
HSV-1 + - + + - -
Control Ab - - - + - +
Anti-FasL - - + - + -

Figure 4

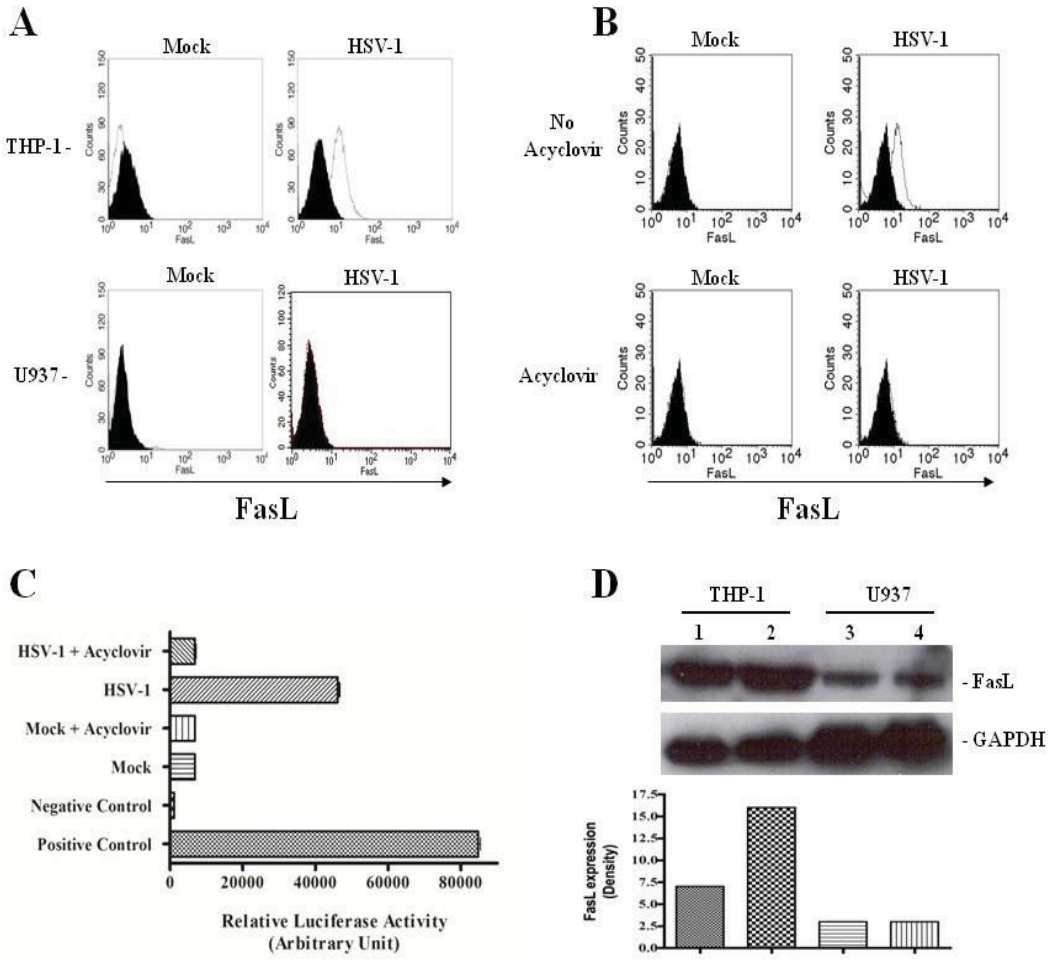

A
Mock
HSV-1
THP-1 -
U937 -
Counts
FasL
B
No Acyclovir
Acyclovir
C
HSV-1 + Acyclovir
HSV-1
Mock + Acyclovir
Mock
Negative Control
Positive Control
0
20000
40000
60000
80000
Relative Luciferase Activity (Arbitrary Unit)
D
THP-1
U937
1
2
3
4
- FasL
- GAPDH
FasL expression (Density)

Figure 5

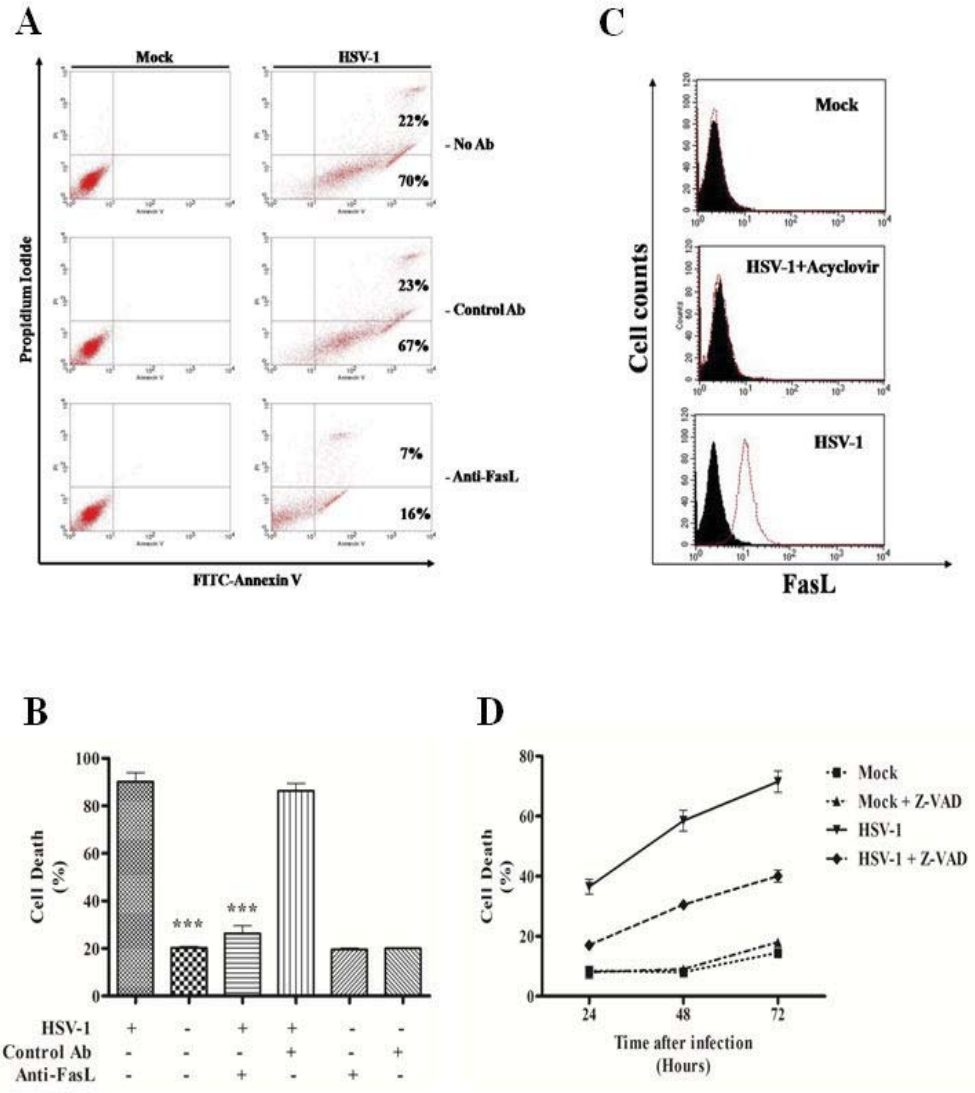

A
Mock
HSV-1
22%
70%
- No Ab
23%
67%
- Control Ab
7%
16%
- Anti-FasL
Propidium Iodide
FITC-Annexin V
C
Mock
HSV-1+Acyclovir
HSV-1
Cell counts
FasL
B
Cell Death (%)
***
***
HSV-1 + - + + - -
Control Ab - - - + - +
Anti-FasL - - + - + -
D
Cell Death (%)
Mock
Mock + Z-VAD
HSV-1
HSV-1 + Z-VAD
Time after infection (Hours)

Figure 6

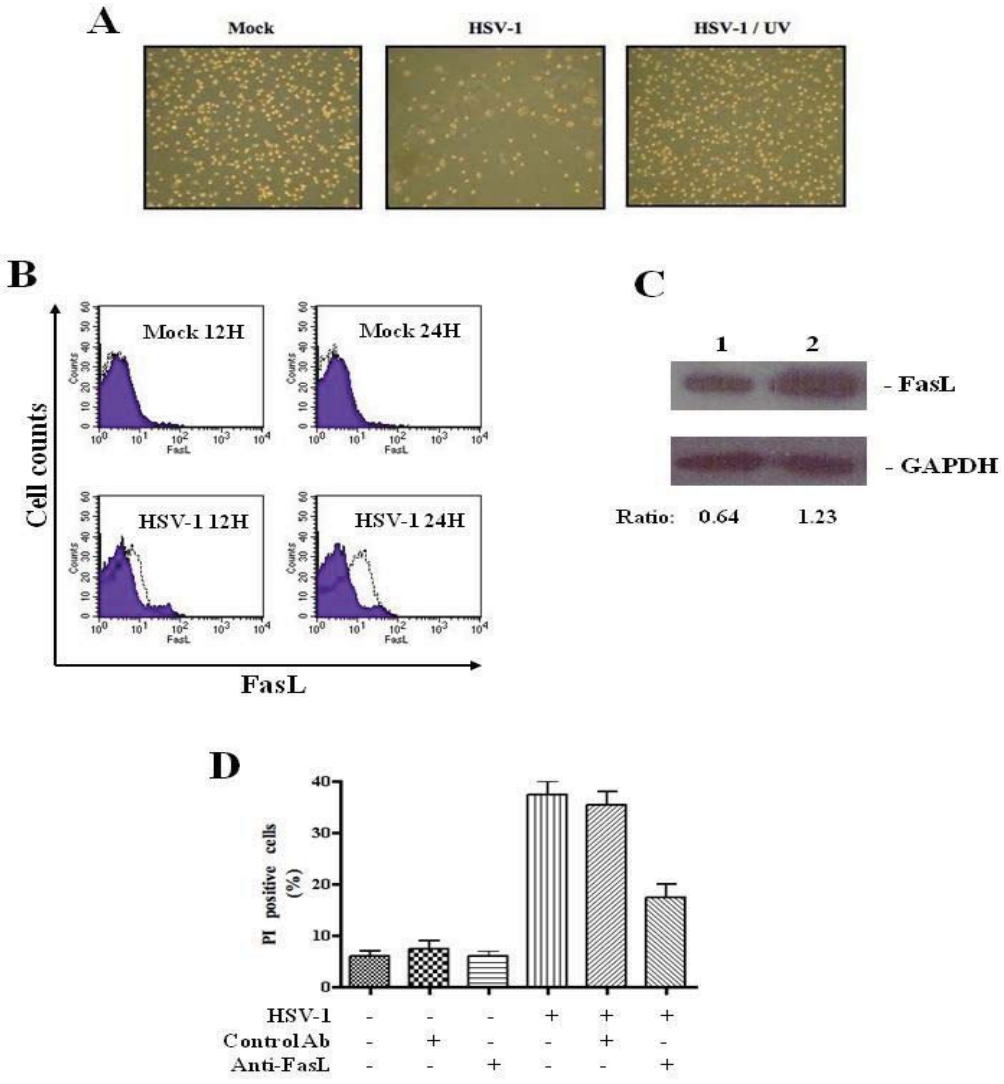
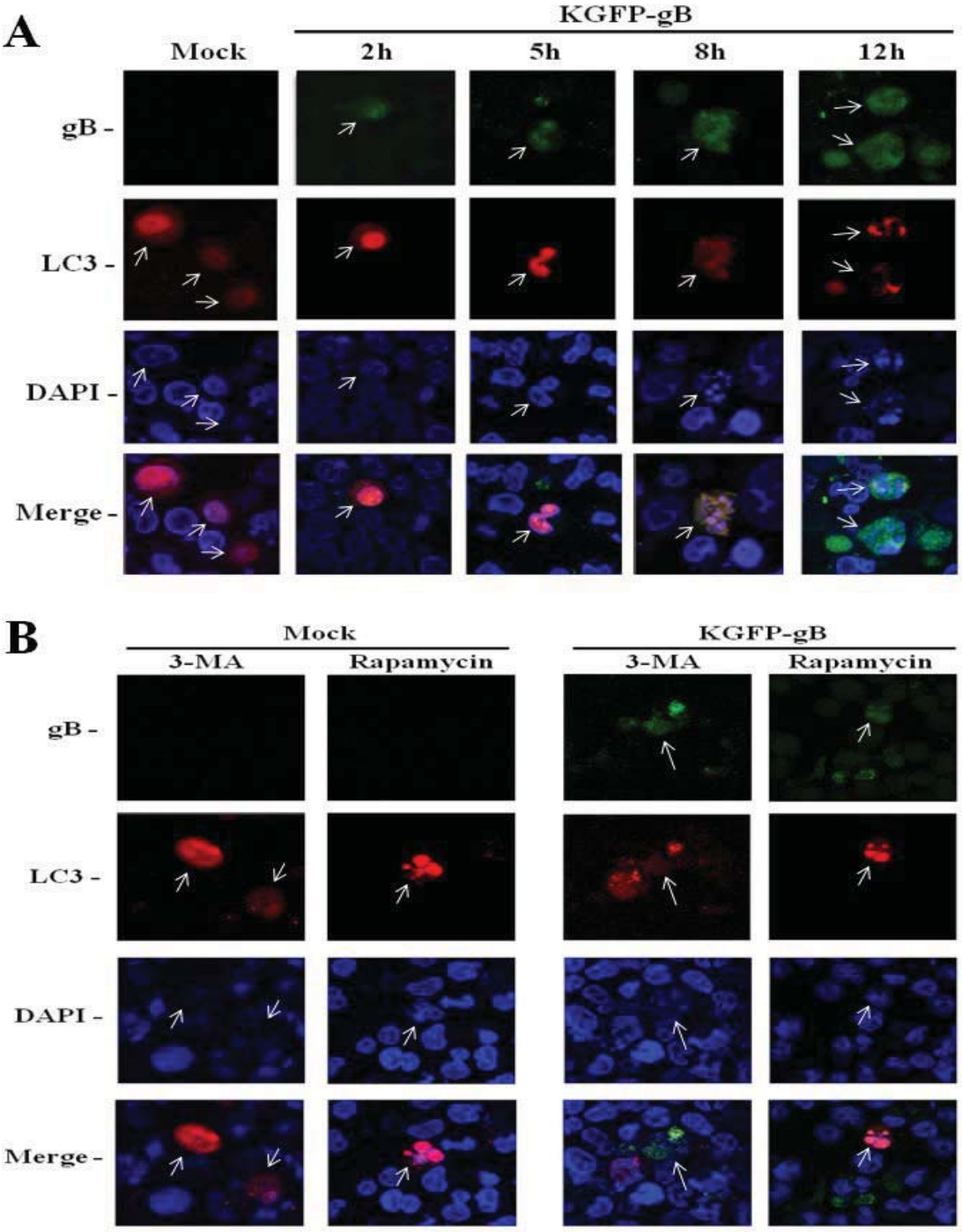

A
Mock
HSV-1
HSV-1 / UV
B
Mock 12H
Mock 24H
HSV-1 12H
HSV-1 24H
Cell counts
FasL
C
1
2
- FasL
- GAPDH
Ratio: 0.64 1.23
D
PI positive cells (%)
HSV-1
ControlAb
Anti-FasL

Figure 7

Figure 8


A
KGFP-gB
Mock
2h
5h
8h
12h
gB -
LC3 -
DAPI -
Merge -
B
Mock
KGFP-gB
3-MA
Rapamycin
3-MA
Rapamycin
gB -
LC3 -
DAPI -
Merge -

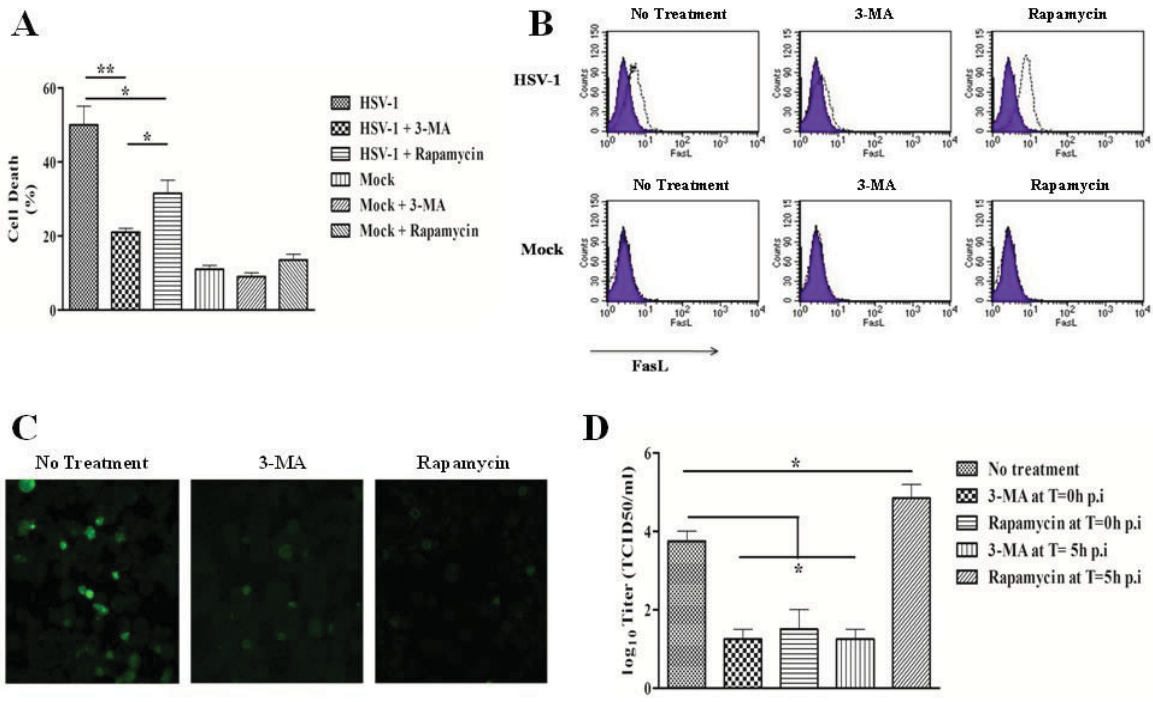

A
Cell Death (%)
HSV-1
HSV-1 + 3-MA
HSV-1 + Rapamycin
Mock
Mock + 3-MA
Mock + Rapamycin
B
No Treatment
3-MA
Rapamycin
HSV-1
Mock
FasL
C
No Treatment
3-MA
Rapamycin
D
log10 Titer (TCID50/ml)
No treatment
3-MA at T=0h p.i
Rapamycin at T=0h p.i
3-MA at T= 5h p.i
Rapamycin at T=5h p.i

Figure 9